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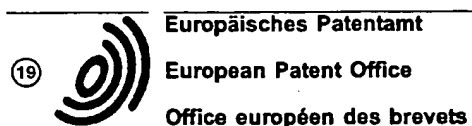
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(11) Publication number : **0 509 841 A2**

(12) **EUROPEAN PATENT APPLICATION**

(21) Application number : **92303492.0**

(51) Int. Cl.<sup>5</sup> : **C12N 15/81, C12N 15/62,  
C12N 15/61, C12N 15/14,  
C12P 21/02, C07K 15/00**

(22) Date of filing : **16.04.92**

<p>(30) Priority : <b>18.04.91 JP 114074/91 30.10.91 JP 311601/91</b></p> <p>(43) Date of publication of application : <b>21.10.92 Bulletin 92/43</b></p> <p>(84) Designated Contracting States : <b>CH DE FR GB LI</b></p> <p>(71) Applicant : <b>Tonen Corporation 1-1, Hitotsubashi, 1-Chome Chiyoda-Ku Tokyo (JP)</b></p> <p>(72) Inventor : <b>HAYANO, Toshiya c/o TONEN CORPORATION 1-3-1 Nishi-Tsurugaoka, Ooi-machi Iruma-gun, Saitama-ken (JP)</b></p>	<p>Inventor : <b>Katoh, Setsuko c/o TONEN CORPORATION 1-3-1 Nishi-Tsurugaoka, Ooi-machi Iruma-gun, Saitama-ken (JP)</b></p> <p>Inventor : <b>TAKAHASHI, Nobuhiro c/o TONEN CORPORATION 1-3-1 Nishi-Tsurugaoka, Ooi-machi Iruma-gun, Saitama-ken (JP)</b></p> <p>Inventor : <b>SUZUKI, Masanori c/o TONEN CORPORATION 1-3-1 Nishi-Tsurugaoka, Ooi-machi Iruma-gun, Saitama-ken (JP)</b></p> <p>Inventor : <b>HONMA, Keiichi c/o TONEN CORPORATION 1-3-1 Nishi-Tsurugaoka, Ooi-machi Iruma-gun, Saitama-ken (JP)</b></p> <p>(74) Representative : <b>Nicholls, Kathryn Margaret et al Mewburn Ellis, 2 Cursitor Street London EC4A 1BQ (GB)</b></p>
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(54) **Co-expression system of protein disulfide isomerase gene and useful polypeptide gene and process for producing the polypeptide using its system.**

(57) The present invention is directed to a transformant comprising the following expression units in a co-expressible state :

- an expression unit containing a gene coding for a receptor protein ERD2 from yeast or analog thereof which is capable of binding to a protein localizing in endoplasmic reticulum and having a signal for staying therein ;
- an expression unit containing a gene coding for said protein localizing in endoplasmic reticulum ;
- and
- an expression unit containing a foreign gene coding for a polypeptide which is a subject of function of said protein localizing in endoplasmic reticulum, and

to a transformant comprising, in a co-expressible state, a fusion gene which is composed of a DNA fragment coding for a human serum albumin prepro-sequence and a foreign gene coding for a useful polypeptide.

The present invention is also directed to a process for producing said polypeptide by co-expressing said genes in said transformant such that the polypeptide is predominantly secreted out of the transformant cell. Consequently, the invention has an advantage of improving the productivity of said polypeptide.

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This invention relates to a co-expression system which comprises a gene coding for protein disulfide isomerase (PDI) and a foreign gene coding for a useful polypeptide and to a process for the production of said polypeptide using said system. PDI is an enzyme which enhances formation of the higher-order structure of polypeptides through its function of catalyzing the exchange reaction of a disulfide bond(s) in the polypeptides.

Studies on the *in vitro* refolding of denatured proteins have revealed the presence of both isomerization reactions of a disulfide bond and of a proline peptide as factors for determining a folding rate of polypeptides (Freedman, *Cell*, vol.57, pp.1069 - 1072, 1989; Fisher and Schmid, *Biochemistry*, vol.29, pp.2205 - 2214, 1990). As enzymes which catalyze these slow reactions during the polypeptide folding, peptidyl prolyl cis-trans isomerase (PPI) has been found in the latter case, and protein disulfide isomerase (PDI) and thioredoxin in the former case. According to *in vitro* experiments, these enzymes rise a refolding rate of denatured proteins, thus indicating a possibility of applying them to the *in vitro* refolding of inactive proteins produced by genetic engineering techniques (Schein, *BioTechnology*, vol.7, pp.1141 - 1148, 1989; J. Udaka, *Nippon Nogei Kagaku Kaishi*, vol.64, pp.1035 - 1038, 1990).

Since PDI is soluble in water and can be isolated relatively easily from the liver of mammals, its properties as a catalyst have been studied in detail. PDI catalyzes the exchange reaction between thiol/disulfide bonds and is capable of undergoing formation, isomerization or reduction of the disulfide bond in protein substrates (Freedman, *Cell*, vol.57, pp.1069 - 1072, 1989). It is known that, *in vitro*, PDI enhances the formation or exchange reaction of the disulfide linkage(s) in molecules of a single domain protein such as RNase and of a multiple domain protein such as serum albumin, or enhances the formation of an intermolecular disulfide bond(s) in a protein having a subunit structure such as immunoglobulin, procollagen or the like (Freedman, *Nature*, vol.329, p.196, 1987).

PDI from mammals exists usually as a homodimer of the polypeptide having a molecular weight of about 57,000 and shows a highly acidic pI value (4.2 to 4.3).

The PDI gene from rat liver has been isolated. The amino acid sequence deduced from the DNA sequence of the PDI gene indicated that PDI has an intramolecular duplicate structure consisting of two homologous units. One of these two homologous units has a homology to the amino acid sequence of thioredoxin, indicating that its active has an amino acid sequence similar to that of thioredoxin (Edman *et al.*, *Nature*, vol.317, pp.267 - 270, 1985). Thioredoxin enhances the reduction of a disulfide bond in insulin and the exchange reaction of a disulfide bond in RNase *in vivo*, which indicate that thioredoxin plays a similar role to PDI in the *in vivo* folding process proteins (Pigiet and Schuster, *Proc. Natl. Acad. Sci., USA*, vol.83, pp.7643 - 7647, 1986).

Although the amount of PDI present in a mammalian living body differs depending on the type of tissues and the differentiation stage of cells, such a difference is correlated with the existence of certain secretory proteins. In addition, PDI is localized abundantly in the endoplasmic reticulum through which a protein is known to pass during its secretion. On the basis of these facts, it is assumed that PDI concerns with the formation of a disulfide bond(s) in secretory proteins newly synthesized within cells. Such an assumption is supported by the results of a study on the biosynthesis of  $\gamma$ -gliadin in a cell-free protein synthesis system, that the formation of a disulfide bond in conjunction with the translation of  $\gamma$ -gliadin hardly occurs when an endoplasmic reticulum fraction from which PDI was washed out in advance is used, while the disulfide bond formation is restored by the addition of PDI (Bulleid and Freedman, *Nature*, vol.335, pp.649 - 651, 1988).

In addition to the disulfide bond formation, PDI concerns with other post-translational modifications of proteins. For example, the polyfunctional property of PDI in connection with the protein modifications has been suggested on the basis of its homology to a catalytic unit,  $\beta$ -subunit, of prolyl-4-hydroxylase which catalyzes hydroxylation of proline residues in collagen, to a glycosylation site binding protein that recognizes a signal Asn-X-Ser/Thr of a peptide to which a sugar chain is bound during N-glycosylation process of synthetic protein (Pihlajaniemi *et al.*, *EMBO J.*, vol.6, pp.643 - 649, 1987; Geetha-Habib *et al.*, *Cell*, vol.54, pp.1053 - 1060, 1988), to a thyroid hormone binding protein (triiodo-L-thyronine binding protein; Cheng *et al.*, *J. Biol. Chem.*, vol.262, pp.11221 - 11227, 1987), etc. In addition to these facts, some molecular species having partly homologous amino acid sequences to PDI have been found though different from the PDI. For example, certain gonadotropic hormones such as follitropin and lutropin contain amino acid sequence homologous to an amino acid sequence which is regarded as an active site of PDI, and these hormones catalyze the isomerization of a disulfide bond (Boniface *et al.*, *Science*, vol.247, pp.61 - 64, 1990). Also, phospholipase C, an enzyme which hydrolyzes phosphatidylinositol-4,5-bisphosphate into 1,2-diacyl glycerol and inositol-1,4,5-triphosphate, has a domain homologous to PDI in its molecule (Bennett *et al.*, *Nature*, vol.334, pp.268 - 270, 1988). In consequence, PDI and PDI-like molecules seem to concern in a markedly wide range of vital phenomena, both intracellularly and extracellularly.

Although the PDI has extensive functions as described above, a main effect of PDI is to form a protein (or a protein aggregate) having a natural higher-order structure by catalyzing the isomerization of an intramolecular or intermolecular disulfide bond(s). In many cases, however, an almost stoichiometric amount of PDI is required

to attain an optimum reaction rate. It is expected therefore that an intramolecular or intermolecular isomerization rate of a disulfide bond will be slow when a disulfide isomerase has a low activity, and such a slow reaction rate will entail a low formation efficiency of a protein having a suitable disulfide bond(s). It is thought that such a low disulfide isomerase activity is one of the cause of the formation of insoluble molecular aggregates of various eukaryote-originated proteins (especially secretory proteins) in *Escherichia coli*. Although *E. coli* contains thioredoxin which is superior to PDI in terms of the activity as a disulfide reductase, the isomerase activity of thioredoxin is low. On the contrary, since an intramolecular disulfide bond(s) can often be found in secretory proteins, it is thought that a disulfide bond activity resulted from disulfide isomerization is high in cells or tissues which have a high secretion ability. This was indicated strongly by a comparative study on the relative PDI mRNA contents in various rat tissues, in which the contents in organs were found to be liver > pancreas, kidney > lung > sperm, spleen > heart > brain in order (Edman *et al.*, *Nature*, vol.314, pp.267 - 270, 1985).

When a reduced condition is given to a given synthesis system, the formation of a disulfide bond which is necessary for the suitable folding of a polypeptide will be inhibited. Such a condition is generated for example in prokaryotic cells which have no compartments. Taking this into consideration, prokaryotic cells and eukaryotic cells may be different from each other in terms of factors concerning the formation of a disulfide bond and of conditions which enable its formation. When useful proteins (most of them are secretory proteins) is produced by recombinant DNA techniques, it is necessary to form a disulfide bond under certain conditions which are suitable for each protein to be produced. To accomplish such conditions, a host cell should have a suitable compartment and a large amount of a disulfide-forming enzyme (i.e., disulfide isomerase) which has a high affinity for the compartment.

In eukaryotes, secretory proteins are transported outside the cell through the endoplasmic reticulum, Golgi body and secretory granules, and such a secretion process is regarded as a passive flow which is called "bulk flow". On the other hand, proteins localizing in the cavity of endoplasmic reticulum were initially thought to stay therein via such a process that the corresponding proteins synthesized were incorporated into the endoplasmic reticulum in the similar manner to the secretory protein and then transferred along the "bulk flow", but sent back again from body to the endoplasmic reticulum by a certain mechanism. Thereafter, primary structures of various proteins localizing in the mammalian endoplasmic reticulum have been determined. From some of these proteins, such as a protein disulfide isomerase (PDI), a glucose-regulated protein 78 (grp78, the same as Bip which as an immunoglobulin heavy chain binding protein) and a glucose-regulated protein 94 (grp94), a common C-terminal sequence "KDEL" ("HDEL" in the case of yeast) consisting of 4 amino acid residues was found. In addition, it was suggested that, in yeast cells, this sequence acts as a signal for allowing proteins to localize in the endoplasmic reticulum, because a mutant protein in which grp78 was deleted is secreted extracellularly and because lysozyme, in spite of its secretory nature, can stay in the endoplasmic reticulum when the "HDEL" sequence is bound to its C-terminus (Munro, S. and Pelham, H.R.B., *Cell*, vol.48, p.899, 1987; Pelham, H.R.B., Hardwick, K.G. and Lewis, M.J., *EMBO J.*, vol.7 p.1757, 1988). In consequence, it was considered that an endoplasmic reticulum or Golgi body contains acceptor molecules specific for the "KDEL" or "HDEL" sequence, and that the acceptor controls localization of a protein having such a sequence on its C-terminus.

Thereafter, an acceptor for the signal "HDEL" was identified in yeast by the analysis of a yeast mutant *erd2* in which a protein having "HDEL" sequence does not stay in the endoplasmic reticulum but transfer along its secretion process, and the same acceptor was also identified in mammals by the analysis in which anti-idiotypic antibodies specific for "KDEL" sequence were used (Semenza, J.C., Hardwick, K.G., Dean, N. and Pelham, H.R.B., *Cell*, vol.61, p.1349, 1990; Vaux, D., Tooze, J. and Fuller, S., *Nature*, vol.345, p.495, 1990). Gene structure of the yeast "HDEL" acceptor has been revealed from which its primary amino acid sequence was deduced, with an estimated molecular weight of 26 kd. On the other hand, the mammalian "KDEL" acceptor identified by the use of anti-idiotypic antibodies has been reported to have a molecular weight of 72 kd, thus indicating the mammalian acceptor is probably different from the above yeast acceptor. Thereafter, a gene coding for a protein homologous to the yeast "HDEL" acceptor has been cloned in mammals by cross-hybridization (Lewis, M.J. and Pelham, H.R.B., *Nature*, vol.348, p.162, 1990). However, it is not clear whether the two different signal acceptors function with mutual relationship or independently in mammals.

In addition to the "KDEL" and "HDEL" sequences, other homologous sequences such as "DEL", "ADEL", "SDEL", "RDEL", "KEEL", "QEDL", "HIEL", "HTEL" and "KQDL" are known as signals for staying in endoplasmic reticulum, and polypeptides having these sequences are considered to stay in endoplasmic reticulum by associating with the aforementioned acceptor molecules (Pelham, H.R.B., *TIBS*, vol.15, p.483, 1990).

However nothing is in practice known about an *in vivo* system in which PDI is contained in a large quantity in a suitable compartment in the coexistence of a useful target protein, the PDI being capable of acting on the protein. Moreover, in spite of the applicability of PDI to the *in vitro* refolding of denatured proteins and to the improved productivity of secretory proteins in cells, this enzyme has been prepared only by direct purification from the internal organs. In addition, there are no reports on the interspecific expression of PDI, and on the

establishment of a process for its production by means of genetic engineering or a process in which the productivity of a useful polypeptide is improved by the combination of the PDI gene with a gene coding for the polypeptide.

According to one aspect of the present invention there is provided a transformant comprising the following  
5 expression units integrated on yeast chromosome in a co-expressible state:

an expression unit containing a gene coding for a receptor protein ERD2 from yeast or analog thereof which is capable of associating with (or binding to) a protein localizing in endoplasmic reticulum and having a signal for staying therein; and

an expression unit containing a gene coding for said protein localizing in endoplasmic reticulum and hav-  
10 ing a signal for staying therein.

Preferably, the gene coding for a protein localizing in endoplasmic reticulum and having a signal for staying therein is selected from the group consisting of a human protein disulfide isomerase (PDI) gene and a fusion gene which is composed of a DNA fragment coding for a human serum albumin (HSA) prepro-sequence and the isomerase gene. According to one aspect of the invention, said fusion gene has a base sequence coding  
15 for the -24 to +491 amino acid sequence shown in SEQ ID NO:2.

It is desirable that embodiments of the present invention provide a transformant comprising an expression unit containing a foreign gene coding for a polypeptide which is a subject of function of said protein localizing in endoplasmic reticulum, other than the two expression units described above, the three expression units being integrated on yeast chromosome.

According to another aspect of the present invention there is provided a fusion gene for use in an expression of PDI, which is composed of a DNA fragment coding for a HSA prepro-sequence and a gene coding for the PDI.

Yet another aspect of the present invention provides a process for the production of a polypeptide which comprises co-expressing a human PDI gene and a foreign gene coding for the polypeptide to be produced, in  
25 the above-described transformant comprising these genes so as to produce the polypeptide. Embodiments of the present invention desirably further provide a process for the production of a polypeptide which comprises secreting it predominantly out of the transformant cell through the co-expression of said genes.

Embodiments of the invention are described below by way of example only and with reference to the figures of which:

30 Fig. 1 illustrates a construction of the expression plasmid pAHhPDILyI.

Fig. 2 shows a boundary of the HSA prepro-sequence and the PDI gene on an expression plasmid.

Fig. 3 is a photograph showing the result of SDS-polyacrylamide gel electrophoresis of an expressed and secreted crude recombinant human PDI, wherein lane 1 is a molecular weight marker, lane 2 is pAH/AH22 (control) and lane 3 is pAHhPDILyI/AH22.

35 Fig. 4 illustrates the separation of a recombinant human PDI by hydrophobic column chromatography.

Fig. 5 shows the result of SDS-polyacrylamide gel electrophoresis of a purified recombinant human PDI, wherein the numbers at the bottom correspond to the fraction numbers of the hydrophobic column chromatography shown in Fig. 4, and M is a molecular weight marker.

Fig. 6 is a photograph of SDS electrophoresis showing expression of human PDI in the yeast strain HIS23.

40 Fig. 7 is a photograph of SDS-polyacrylamide gel electrophoresis showing secretion of HSA by co-expression of human PDI and HSA in the yeast strain HIS23.

Fig. 8 shows the result of densitometric determination of the amount of secreted HSA using the SDS-polyacrylamide gel electrophoresis gel of Fig. 7.

Fig. 9 illustrates a preparation of the *Xho*I-*BAM*HI restriction fragment of YERD2.

45 Fig. 10 illustrates a construction of the expression plasmid pIVTRPGAPYERD2.

Fig. 11 illustrates a construction of the expression plasmid pIVTRPADHYERD2.

Fig. 12 is a photograph showing the result of SDS-polyacrylamide gel electrophoresis of human PDI and HSA which were secreted from the strain SN35A-1PU capable of expressing both human PDI and HSA and from the strain SN35A-1PUAET introduced a yeast ERD2 expression system into the SN35A-1PU, wherein  
50 lane 1 is a supernatant of the culture of SN35A-1PU and lane 2 is a supernatant of the culture of SN35A-1PUAET.

Fig. 13 shows the result of densitometric determination of an amount of HSA secreted from strain SN35A-1PU and SN35A-1PUAET using SDS-electrophoresis gels.

The present invention has been completed by finding a fusion gene for expression of PDI which is composed of a DNA fragment coding for human serum albumin (HSA) prepro-sequence and a gene coding for human PDI, as well as a co-expression system comprising PDI gene, yeast ERD2 gene and a certain polypeptide gene.  
55

The following describes the present invention in detail:

Clones containing a human PDI cDNA are isolated from the human liver and placenta  $\lambda$ gt11 cDNA libraries (Clontech, US) by the following procedures:

An *E. coli* strain infected with phage from the human liver and placenta  $\lambda$ gt11 cDNA libraries, after which DNAs from the phage grown are fixed on a filter. Separately from this, positive clones are screened by hybridization using a 40 mer synthetic oligomer DNA as a probe which corresponds to the complementary strand of a nucleic acid sequence (243-282) of human proline 4-hydroxylase (the same protein as PDI) cDNA (Pihlajaniemi, T. *et al.*, *EMBO J.*, vol.6, p.643, 1987). The phage DNA obtained is digested with *EcoRI*, and the resultant 150 bp insert DNA is used as a probe for screening the PDI cDNA. Using the probe, the phage DNAs fixed on the filter are screened to isolate positive clones.

Thereafter, a plurality of positive clones obtained in such a manner are digested with *EcoRI* so as to isolate *EcoRI* insert DNA fragments, and a restriction map of the insert of each clone is made. From the comparison of these maps with that reported by Pihlajaniemi *et al.*, it was estimated that the full length human PDI cDNA was recovered by a clone (pHPDI16) from liver and a clone (pHPDIp4) from placenta.

Determination of DNA sequences of the two clones revealed that these clones encoded human PDI cDNA consisting of 2454 base pairs in full length as shown in the SEQ ID No:1. An amino acid sequence deduced from the DNA sequence is also shown in the SEQ ID No:1. In the amino acid sequence, a mature protein seems to be composed of 491 amino acids from Asp<sup>1</sup> to Leu<sup>491</sup>, and the 17 amino acid polypeptide preceding Asp<sup>1</sup> seems to be a signal peptide.

According to the present invention, there is provided a fusion gene for use in the expression and production of PDI, which is composed of a DNA fragment coding for a HSA prepro-sequence and the aforementioned human PDI gene.

As shown in Fig. 1, the fusion gene is constructed in general by arranging the preprosequence-encoding DNA fragment at the upstream side of the PDI gene. In this instance, however, a leader sequence for transporting human PDI into an appropriate compartment (considered to be endoplasmic reticulum) is not always limited to the HSA prepro-sequence, and other signal sequences or prepro-sequences may also be used as the leader sequence.

More particularly, said fusion gene may be prepared as follows:

The aforementioned clones pHPDI16 and pHPDIp4 DNAs are double-digested with *EcoRI*/*PstI* and *PstI*/*BamHI*, respectively, to produce DNA fragments of about 490 bp and about 1.3 kbp respectively, the fragments recovered are ligated with a plasmid vector pUC119 which was ligated with *EcoRI* and *BamHI* to produce pHPDIEB in which a *NaeI* cleavage site is then introduced into the boundary between the PDI signal sequence and the PDI sequence by the Kunkel's method (Kunkel, T.A., *Proc. Natl. Acad. Sci. USA*, vol.82, p488, 1985) so as to prepare pHPDINae, and thereafter the pHPDINae is digested with *NaeI* and *HindIII* to give a PDI DNA fragment of about 1.7 Kb which does not contain the PDI signal sequence.

Separately from this, pUC119 is digested with *EcoRI*, and the resultant digest is ligated with the following *XhoI* linker:

5' -AATTCTCGAG

GAGCTCTTAA-3'.

After double-digesting the product with *XhoI* and *BamHI*, the digest is ligated with a prepro-sequence of HSA to produce pUC119Sig which is subsequently digested with *SfuI* and *HindIII* to give a DNA fragment of about 3.2 kb. A method for synthesizing the HSA prepro-sequence will be described later in Examples.

Thereafter, the 1.7 kb DNA fragment from pHPDINae and the 3.2 kb DNA fragment from pUC119Sig origin are ligated together to produce pHPDILyI which is in turn digested with *EcoRI*, blunt-ended with Klenow fragment, and digested with *BamHI*, thereby giving a fusion gene in which a leader sequence is modified and in which the human PDI gene is fused to the downstream side of the HSA prepro-sequence (Fig. 2).

Process for the preparation of the fusion gene according to the present invention is not limited to the above-described techniques, provided that said fusion gene has an ability for expressing PDI. Although analogs of the inventive fusion gene are not included within the scope of the present invention, it is obvious that they can be prepared easily from a corresponding gene of any animal origin other than human.

According to one embodiment of the present invention, said fusion gene has a DNA sequence coding for the -24 to +491 amino acid sequence shown in the SEQ ID NO. 2. Instead of the PDI gene therein, a DNA sequence coding for the +1 to +491 amino acid sequence (Asp<sup>1</sup>--Leu<sup>491</sup>) shown in SEQ ID NO:1 may also be applied. In these instances, all genes which substantially have the same function as that of said DNA sequences, for example, derivatives having nucleotide sequences based on the degeneracy of codon, are included within

the scope of the present invention. According to another embodiment of the present invention, an example of such a fusion gene includes the sequence between nucleotide 1 to nucleotide 1545 shown in SEQ ID NO.2.

An expression vector used for the insertion of the linked gene of the present invention thereinto should replicate in a host cell and have the ability for expressing therein. In general, such a useful vector contains replicon, and regulatory sequences which are derivated from a species compatible with a host to be used, as well as a replication origin and a marker sequence which enable selection of a phenotype from transformed cells.

As a vector for use in the construction of the expression vector, the plasmid pJDB-ADH-HSA-A (Fig. 1) which has been disclosed in Japanese Patent Application Laying-Open (KOKAI) No. 2-117384 filed by the present applicant may be used conveniently. This plasmid contains HSA cDNA, as well as yeast alcohol dehydrogenase I (ADH I) promoter, ADH I terminator, ampicillin resistance gene (Amp<sup>r</sup>) and Leu2 gene. The HSA cDNA is removed from this plasmid by digesting it with *Xho*I, blunting with Klenow fragment, and then digesting with *Bam*HI. The 5'-end of the DNA fragment of about 8 kb thus obtained is dephosphorylated, and the resultant fragment is ligated with the aforementioned fusion gene of the present invention to give the expression vector pAHhPDILyl. In this process, other type of vectors can be used provided that they are capable of expressing the fusion gene.

Examples of host for use in the expression of human PDI include prokaryotes such as *E. coli*, *Bacillus subtilis*, etc and eukaryotes such as yeast, etc. Preferred is a host cell capable of secreting the mature PDI via processing. Preferably, the host cell is such as *Saccharomyces cerevisiae*, more preferably yeast strain AH22. It is obvious that eukaryotes other than yeast, for example animal cells, can be used as the host cell. Incorporation of the expression vector into a host cell can be carried out easily by conventional means such as calcium chloride, protoplast (or spheroplast)-polyethylene glycol, electroporation, etc. When the plasmid pAHhPDILyl is used as an expression vector, a desired transformant may be obtained by culturing transformant cells on SD(-Leu) plate and screening colonies grown on the plate.

A process for the production of a recombinant type human PDI comprises the steps of:

- constructing an expression vector which can replicate in a host cell and express the fusion gene of the present invention therein;
- isolating a host cell transformed with said expression vector;
- culturing the obtained transformant under such conditions that the fusion gene can be expressed, thereby secreting said recombinant human PDI; and
- recovering the recombinant PDI.

The recombinant human PDI can be purified easily by separating the transformed cells from a cultured medium by centrifugation, disrupting the cells if necessary, concentrating the supernatant by ultrafiltration or the like, and then subjecting the concentrate to hydrophobic column chromatography. Through not particularly limited, TSK-gel Phenyl-5PW hydrophobic column (Tosoh, Japan) may be used in the chromatography. In this case, the recombinant human PDI may be eluted by linear gradient of from 0.85 to 0 M ammonium sulfate in Borate buffer (Ph 8.0) containing KCl (Fig. 4). It was confirmed that the purified recombinant human PDI has a molecular weight of about 55 kDa based on the SDS-polyacrylamide gel electrophoresis analysis (Fig. 5), and practically has PDI activity as the results of determination of a degree of the refolding of scrambled ribonuclease A (see Examples).

In comparison with the natural type human PDI, it has been found that the recombinant human PDI thus prepared has the same amino acid sequence except that its N-terminal amino acid is changed from Asp to Gly, as shown in SEQ ID NO:3.

The present invention further provides a transformant comprising a fusion gene which is composed of a human PDI gene and a DNA fragment coding for a HSA prepro-sequence, and a foreign gene coding for a polypeptide to be produced, in a co-expressible state.

The fusion gene and the foreign gene in the transformant may be located on the same or different chromosome(s), provided that they are mutually present in a co-expressible state. Transformation of a host cell can be carried out for example by inserting the fusion gene and the foreign gene into the same or different vector(s) and introducing the resulting vector into the host cell by conventional means as lithium chloride, protoplast (or spheroplast)-polyethylene glycol, electroporation, etc.

The foreign gene may encode a polypeptide of any type, provided that the polypeptide contains at least one disulfide linkage because the catalyst effect of an amplified and expressed PDI, that is acceleration of the formation or exchange reaction of a disulfide bond(s) in polypeptide, is directly exhibited. In addition, the present invention can be applied to a case which the PDI activity exerts influence on proteins relating to gene expression, polypeptide folding or transport, thereby indirectly improving the productivity of PDI. According to the embodiment of the present invention, the foreign gene is gene coding for HSA.

The term "polypeptide" as used herein means a short- or long-chain peptide and protein.

Examples of hosts include prokaryotes such as *E. coli*, *Bacillus subtilis*, etc and eukaryotes such as yeast,

animal cell, etc. Preferred is a host cell capable a mature polypeptide through post-translational modification and processing, more preferably eukaryotes, and most preferably yeast.

The present invention also provides a process for producing a polypeptide, which comprises the following steps of:

- 5 co-expressing a human PDI gene and a foreign gene coding for the polypeptide to be produced, in the above-described transformant so as to produce the polypeptide; and
- recovering the polypeptide.

When HSA and PDI are co-expressed within an HSA-producing yeast strain (pAHhPDILy1/HIS23) transformed with a human PDI expression plasmid in an appropriate medium, a secretion level of HSA practically increase by about 60% in average in comparison with the case of a non-transformed HSA-producing yeast strain (pAH/HIS23) (Fig. 8).

Although we do not intend the present invention to restrict by theory, the increase in the secretion level of HSA by co-expression can be explained as follows:

HSA is a protein containing 17 disulfide bonds. It is known also that formation of its higher-order structure is enhanced in the presence of a stoichiometric amount of PDI in *in vitro* refolding experiments of a denatured protein.

HSA is secreted from the yeast strain HIS23 as a water-soluble molecule, but some of the HSA molecules are also detectable within the yeast cell. When the intracellular HSA was analysed by SDS-polyacrylamide gel electrophoresis, it was detected as a single band with the same mobility as that of a normal HSA molecule in the presence of a reducing agent, while detected as discontinuous bands having a large molecular weight than normal HSA in the absence of a reducing agent, clearly showing a different behavior from that of normal HSA. These results indicate that the presence of intracellular HSA molecules is based upon the incomplete formation of an intracellular disulfide bond(s). In a yeast strain allowed PDI to co-express together with HSA, however, an intracellular HSA molecule was detected as a more narrow band on a SDS-polyacrylamide gel electrophoresed without a reducing agent when compared with an intracellular HSA sample prepared from a yeast strain which can not co-express a foreign PDI cDNA together with HSA. This indicate that PDI enhances the formation of a normal disulfide bond(s) in the HSA molecule and thereby assists the formation of the higher-order structure of HSA molecule more efficiently. Accordingly, it is suggested that the co-expression of PDI reduces chances of causing the association of HSA molecules having unstable structure and their decomposition by proteases in the host cell, thereby increasing the secretion of HSA molecules.

When the amount of HSA mRNA in the HIS23 in which PDI was co-expressed is compared with the amount in a control without co-expression by means of northern blotting, increase in the amount of HSA mRNA can be found in the former cells in which the PDI gene was expressed. These results suggest that PDI exerts influence not only directly on HSA molecules but also on the transcription level of the HSA gene. However, it seems to be reasonable that the increment of HSA production level is base on the direct influence of PDI on HSA molecules by their coexistence in endoplasmic reticulum, because the increase in the amount of secreted HSA has a correlation to an increased level of the secretion of human PDI out of the yeast cell based upon the fusion of the PDI gene with the HSA prepro-sequence which plays a role in the intracellular transport into endoplasmic reticulum through a transmembrane process. In addition, when the amounts of the HSA and PDI secreted from the HIS23 cells are compared with each other, PDI is secreted in several times larger amounts than HSA, and the level of human, PDI detectable within the cells is also higher than HSA. These results, therefore, indicate that PDI is localized in the endoplasmic reticulum of the yeast cells in an enough amount to enhance the *in vitro* refolding of a denatured HSA, which also supports the direct effect of PDI on HSA.

Thus it is highly possible that the effect of the co-expression of PDI on an increment of the amount of secreted HSA is based on the direct influence of PDI on the formation of the higher-order structure of HSA. In consequence, such a similar secretion-improving effect can also be expected in other general secretory proteins in which the formation of a disulfide bond(s) contributes to the formation and stability of their higher-order structures, by highly amplified co-expression of PDI in the same host cell.

The present invention also provides a transformant comprising the following expression units integrated on yeast chromosome in a co expressible state:

- an expression unit containing a gene coding for a receptor protein ERD2 from yeast or analog thereof which is capable of binding to a protein localizing in endoplasmic reticulum and having a signal for staying therein; and
- an expression unit containing a gene coding for said protein localizing in endoplasmic reticulum and having a signal for staying therein.

Such a transformant is useful for the preparation or another transformant cell capable of secreting a useful polypeptide predominantly out of a transformant, by transforming its host cell with an expression vector which contains a foreign gene coding for the polypeptide, said polypeptide being a subject of function of the aforesaid



protein localizing in endoplasmic reticulum.

The wording "protein localizing in endoplasmic reticulum and having a signal for staying therein" as used herein is intended to include any protein which can localize in the endoplasmic reticulum cavity after *in vivo* protein synthesis, which has a signal for staying in endoplasmic reticulum, such as amino acid sequence "KDEL", "HDEL", "DDEL", "ADEL", "SDEL", "RDEL", "KEEL", "QEDL", "HIEL", "HTEL", "KQDL" and the like, on the C-terminus of the protein, and which is capable of binding to a receptor protein ERD2 from yeast or analog thereof. Examples of such a type of proteins include PDI, glucose-regulated protein 78 (grp78), glucose-regulated protein 94 (grp94), etc. Preferred is a protein having a useful function for a polypeptide, more preferably PDI. The PDI is known as an enzyme which catalyzes the exchange reaction of a thiol/disulfide bond and rises a rate of the refolding of a denatured protein (Schein, *Bio/technology*, vol.7, pp. 1141 - 1148, 1989; Freedman, *Cell*, vol.57, pp.1069 - 1072, 1989). According to the present invention, the above-mentioned protein localizing in endoplasmic reticulum also includes a protein which has no said signal natively on its C-terminus but has been modified by genetic engineering techniques so as to provide it with the signal.

The wording "acceptor protein ERD2 from yeast or analog thereof" as used herein means an acceptor protein from yeast which is capable of accepting any protein localizing in endoplasmic reticulum and having a signal for staying therein. An example of the DNA sequence encoding the ERD2 protein is a sequence reported by Semenza *et al.* (Semenza, J.C., Hardwick, K.G., Dean, N. and Pelham, H.R.B., *Cell*, vol.61, p.1349, 1990) which is included herein as reference, although any other modified DNA sequence may also be included provided that the modification does not spoil the function of the ERD2. Such a modified DNA sequence is, for example, a DNA sequence coding for a "KDEL" acceptor from mammals which has a high homology to the ERD2 protein (Lewis, M.J. and Pelham, H.R.B., *Nature*, vol.348, p.162, 1990).

The following describes a process for the preparation of an expression vector which comprises a gene coding for the yeast ERD2. It should be understood, however, that the vector obtained by the process is a representative one and that it is not intended to limit to the vector alone.

A gene encoding the yeast ERD2 is obtained by the polymerase chain reaction (PCR) technique (Mullis, K.B. and Faloona, F., *Meth. Enzymol.*, vol.155 p.335, 1987) using a genomic DNA prepared from the yeast strain S288C as a template. Primers used are : 5'-TTTTTCTCGAGTAAGCAATGAATCCGTT-3' and 5'-AAAAAGGATCCTGCAACACTATTAAA-3', which were designed based on the DNA sequence of the yeast ERD2 gene (Semenza, J.C., Hardwick, K.G., Dean, N. and Pelham, H.R.B., *Cell*, vol.61, p.1349, 1990). The ERD2 gene obtained is inserted into a *XhoI/BamHI* site of the plasmid vector BluescriptII SK+ and then subcloned (Fig. 9).

A vector for use in the incorporation of the ERD2 gene is capable of expressing said gene in a host cell and of replicating itself therein along with the replication of the host chromosome after its integration on the chromosome. In general, the vector contains a marker sequence which enables selection of a phenotype from transformed cells.

As a vector for use in a construction of the expression vector of the present invention, plasmid pRG-UAS1-N7-TLY1-304 may be used conveniently (A procedure for constructing the plasmid will be described in detail in Example.). This plasmid contains human serum albumin (HSA) cDNA, as well as a modified promoter derived from a yeast glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter, a yeast alcohol dehydrogenase I (ADH I) terminator, an ampicillin resistance gene (*Amp<sup>r</sup>*) and a TRP1 gene. The HSA cDNA is removed from this plasmid by digesting it with *XhoI* and *BamHI*, which is then ligated with the yeast ERD2 gene to give the vector pIVTRPGAPYERD2 (Fig. 10). Thereafter, the pIVTRPGAPYERD2 can be converted into expression plasmid pIVTRPADHYERD2 by digesting pIVTRPGAPYERD2 with *HindIII* and *XhoI* to remove the modified GAP promoter and by ligating the resultant fragment with a yeast ADH promoter (Fig. 11). Any other type of vectors which function for the expression of the ERD2 gene may be used instead of the pRG-UAS1-N7-TLY1-304.

In addition to the alcohol dehydrogenase I promoter, examples of other promoter sequences useful in the expression vector include 3-phosphoglycerate kinase promoter (Hitzenman *et al.*, *J. Biol. Chem.*, vol.225, p.2073, 1980) and promoters for the glycolytic enzymes such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, etc (Hess *et al.*, *J. Adv Enzyme Reg.*, vol.7 p.149, 1968; Holland *et al.*, *biochemistry*, vol.17, p.4900, 1978). Any terminator sequence compatible with a yeast strain may be used in the expression vector, examples of which are terminators for the above enzymes.

The expression vector may further contain a yeast-originated gene such as the TRP1. This gene can be used as a phenotype selection marker when a desired transformant is isolated, and it can also function in order to occur the homologous recombination of an ERD2 expression unit on yeast chromosome when the vector is integrated onto the chromosome. The expression vector may also contain a yeast-compatible replication origin, a ribosome binding site, a marker sequence such as antibiotic resistant gene, and other useful sequences.

A preferred example of the above-mentioned gene coding for a protein localizing in endoplasmic reticulum

and having a signal for staying therein, is a PDI gene or a fused gene which is composed of the PDI gene and a HSA prepro-sequence. Examples of the PDI gene used in the present invention include an eukaryote PDI gene, particularly mammal PDI gene, more particularly human PDI gene, and its derivatives (substitution, addition, modification, deletion, etc). The human PDI gene or its derivative has for example a DNA sequence coding for the amino acid sequence shown in SEQ ID NO:1 or SEQ ID NO:3. With respect to the construction of a PDI expression unit, the above descriptions about the construction of ERD2 expression system are applied thereto directly. Its illustrative procedures will be described in Examples.

Although not particularly limited, a strain belonging to the genus *Saccharomyces*, such as *Saccharomyces cerevisiae*, may be used as a yeast host. It is obvious that eukaryotes other than yeast, for example animal cells, can also be used as the host.

Transformation can be carried out easily by conventional means such as lithium chloride, protoplast(or spheroplast)-polyethylene glycol, electroporation, etc.

When the transformant of this invention contains TRP1 gene, it may be isolated by culturing the transformed cell on an SD (-Leu, -His, -Ade, -Ura, -Trp) plate and screening colonies on the plate.

The present invention also provides a transformant comprising the following expression units integrated on yeast chromosome in a co-expressible state:

an expression unit containing a gene coding for a receptor protein ERD2 from yeast or analog thereof which is capable of binding to a protein localizing in endoplasmic reticulum and having a signal for staying therein;

an expression unit containing a gene coding for said protein localizing in endoplasmic reticulum and having a signal for staying therein; and

an expression unit containing a foreign gene coding for a polypeptide which is a object of function of said protein localizing in endoplasmic reticulum and having a signal for staying therein.

The foregoing descriptions concerning the expression unit comprising a gene encoding a ERD2 or analog thereof and the expression unit; the expression unit comprising a gene encoding the aforesaid protein localizing in endoplasmic reticulum; the yeast host; and the transformation process, may directly be applied to this case.

With regard to the polypeptide to be expressed, any type of polypeptides which are susceptible to the PDI activity may be used. According to the embodiment of the present invention, HSA gene may preferably be used as the gene encoding a polypeptide.

The ERD2 gene and other above-described genes which are carried in the two types of the transformants of the present invention may be located on the same or different genome(s) in the host cell, provided that they are mutually present in a co-expressible state. For example, the ERD2 gene and other above-described genes may be inserted into the same vector or preferably different vectors, incorporated into the same host cell, and then integrated into the host chromosome by homologous recombination. Each of the expression units containing the ERD2 gene and other above-described genes may be contained in plural numbers in the chromosome, provided that they are mutually in a co-expressible state.

According to another embodiment of the present invention, the transformant includes the HSA-highly producing yeast SN35A-1 PUAET.

This yeast strain is obtained by incorporating an expression vector containing the yeast ERD2 gene into the SN35A-1PU (obtained by introducing a human PDI expression unit into the locus *ura3* of a HSA-highly secreting yeast strain SN35A (Japanese Patent Application No. 3-2226107); see Examples) and then by integrating the ERD2 gene into the *trp1* site on the yeast chromosome by homologous recombination. The yeast transformant SN35A-1PUAET thus obtained can express the yeast ERD2, human PDI and HSA simultaneously. In comparison with the SN35A-1PU without a ERS2 expression system, the secretion of PDI into a culture medium is repressed significantly, and an average level of the secretion of expressed HSA increases by about 26% after culturing for 24 hours and by about 17% after culturing for 48 hours as compared with the control (Figs. 12 and 13).

The present invention also provides a process for the production of a polypeptide, which comprises the following steps of:

culturing a transformant containing the aforementioned three expression units in an appropriate medium, and bringing about co-expression such that the polypeptide is predominantly secreted out of the transformant cell, the polypeptide being a subject of function of a protein which localizes in endoplasmic reticulum and has a signal for staying therein, while both an acceptor protein ERD2 from yeast or analog thereof which is capable of associating with said protein localizing in endoplasmic reticulum, and said protein as a ligand for the ERD2 remain in endoplasmic reticulum; and

recovering said polypeptide secreted.

According to the process of the present invention, in order to prevent leakage of said protein localizing in endoplasmic reticulum from a host cell, the expression of the ERD2 protein or analog thereof and of the protein

localizing in endoplasmic reticulum are controlled by a suitable regulator sequence.

Although we do not intend to restrict the present invention by theory, both the repressed secretion of PDI and the enhanced secretion of HSA by co-expression of the ERD2 may be explained as follows:

With regard to a retention mechanism of PDI in the endoplasmic reticulum, it is considered generally that the ERD2 localized in the endoplasmic reticulum acts as an acceptor of said signal which is attached to the C-terminus of PDI. When PDI is expressed under control of a strong promoter (ex. modified GAP promoter herein), the amount of intracellularly expressed PDI exceeds the PDI-binding capacity of ERD2, and accordingly the excess PDI molecules "over flow" outside the host cell. Such an "over flow" of PDI has been observed by the present inventors through the study on the expression of human PDI in yeast (Japanese Patent Application No. 2-295017 filed by the present applicant). In consequence, it can be explained that the PDI, the excess of which is secreted out of the yeast cell in the case of SN35A-1PU, remains within the yeast cell in the case of SN35A-1PUAET because the PDI-accepting capacity of ERD2 is enhanced in the endoplasmic reticulum by allowing a large number of ERD2 to co-express under control of the ADH promoter.

In this case, it is assumed that the co-expression of ERD2 improves the aforesaid PDI-retention efficiency in the endoplasmic reticulum, and thereby an amount of secreted HSA as a substrate of PDI, increases.

This indicates strongly that the effect of PDI on the enhanced secretion of HSA is not due to the extracellular stabilization of HSA by PDI, but originated from the PDI activity to catalyze the formation of the higher-order structure (i.e., formation of S-S bond) of HSA in host cell (in the endoplasmic reticulum).

Thus, the improvement of a PDI-retention efficiency in the endoplasmic reticulum by the co-expression of a yeast ERD2 gene provides a system in which the PDI can function maximally by allowing a large quantity of expressed PDI to localize within a suitable intracellular compartment where the PDI can function naturally.

The present invention has a general utility value for the purpose of allowing any polypeptide having a signal for staying in endoplasmic reticulum which is acceptable by ERD2, to work efficiently on a foreign polypeptide as its substrate like the case of PDI.

The present invention has established for the first time a means in which the secretion of PDI is repressed and the effect of PDI to increase the secretion of serum albumin is improved, by constructing a large scale expression system of a yeast ERD2 gene and by applying this system to expression systems of human PDI and HSA. The process of the present invention can be used as a means to improve the above-described retention efficiency in conjunction with a large scale expression system of a protein localizing in the endoplasmic reticulum of an eukaryote, as well as a means to improve a production efficiency of a useful polypeptide by introducing the co-expression system into a production system of the polypeptide which is a substrate of said protein.

The following non-limited examples will be provided to further illustrate the present invention.

### 35 Example 1

#### Cloning of human PDI (protein disulfide isomerase) cDNA

About 100,000 clones of human liver  $\lambda$ gt11 cDNA library (Clontech) were mixed with 500  $\mu$ l of a culture of *E. coli* strain Y1090 which has been precultured overnight at 37°C in LB medium (1% Bacto-trypton, 1% NaCl and 0.5% yeast extract) supplemented with 0.2% maltose. After further adding 5  $\mu$ l of 1 M  $MgCl_2$  solution thereto, the mixture was incubated at 37°C for 10 minutes to infect the *E. coli* cells with the phage particles. The resulting cells were added to 50 ml of an LB top agar medium (LB medium, 10mM%  $MgCl_2$  and 0.7% agarose), and then mixed and inoculated on a LB agar plate (23 cm x 23 cm). After solidifying the top agar medium, the plate was incubated overnight at 37°C so as to grow the phage particles. The phage particles obtained were transferred onto a filter (Hybond-N, Amersham). With the phage-attached side upward, the filter was put 1 minute on a 3MM filter paper (Whatman) which has been soaked in an alkaline solution (0.5 N NaOH and 0.15 NaCl), and then for further 1 minute on the same filter paper which has been soaked in a neutral solution (1 M Tris-HCl (pH 7.5) and 1.5 M NaCl). Thereafter, the filter was washed with 2 x SSC solution (20 x SSC = 3 M NaCl + 0.3 M trisodium citrate), air-dried and then exposed to UV ray for 2 minutes so as to fix the phage DNA on the filter. Using the filter thus obtained, a screening of human PDI cDNA was carried out according to the following procedure:

As a probe to be used, a 40 mer oligomer DNA (5'-TGGCGTCCACCTTGGCCAACCTGATCTCG-GAACCTTCTGC-3') which corresponds to the complementary chain of the 243-282 base sequence of human proline-4-hydroxylase (the same protein of PDI) cDNA (Pihlajaniemi, T. *et al.*, *EMBO J.* vol.6, p.643, 1987) was synthesized using an automatic DNA synthesizer (Model 380B, Applied Biosystems).

The 5'-end of the synthesized DNA was labeled by phosphorylation, by incubating 20 pmoles of the DNA at 37°C for 60 minutes in 50 $\mu$ l of mM Tris-HCl (pH 7.5) buffer containing 10 mM  $MgCl_2$ , 5 mM dithiothreitol,

100 $\mu$ Ci [ $\gamma$ -<sup>32</sup>P] ATP (~ 3000 ci/mmol, Amersham) and 12 units of T4 polynucleotide kinase (Takara Shuzo, Japan). The filter obtained above was soaked at 37°C for 1 hour in the prehybridization solution which consists of 5 x Denhardt solution (100 x Denhardt solution = 2% bovine serum albumin + 2% Ficoll 400 + 2% polyvinyl pyrrolidone), 1 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA (pH 8.0), 0.1% sodium dodecyl sarcosinate and 20  $\mu$ g/ml of an ultrasonic-treated salmon sperm DNA. The filter was further soaked in a hybridization solution (prepared by supplementing the prehybridization solution with about 10<sup>6</sup> cpm/ml of the aforementioned labeled DNA) for 15 hours at 37°C. The resulting filter was washed with 2 x SSC solution at room temperature and then with 2 x SSC + 0.1% sodium dodecyl sarcosinate solution at 42°C for 30 minutes (followed by its exposure to an X-ray film (XAR-5 Kodak) overnight at -80°C. After development of the film, 8 positive signals were detected by the primary screening. Phage particles corresponding to those signals were recovered from the aforesaid plate by cutting it out as gel sections, soaked each of the gel section in 1 ml of SM buffer (100 mM NaCl, 10 mM MgCl<sub>2</sub>, 50 mM Tris-HCl (pH 7.5) and 0.01% gelatin), and left overnight at 4°C so as to recover the phage from the gel into the solution. When the 8 positive phages from the primary screening were further subjected to a second screening under the same conditions as those of the primary screening, only one of them remained as a positive clone. This clone was further subjected to a third screening in order to isolate it as a homogeneous positive clone.

A phage DNA was prepared from the positive clone obtained finally by the method of Leder *et al.* (Leder, P., Tiemeir, D. and Enquist, L., *Science*, vol.196 p.175, 1977). The thus prepared phage DNA (1/5 vol) was digested at 37°C for 1 hour in 50  $\mu$ l of the digestion solution consisting of 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6mM MgCl<sub>2</sub>, 6mM mercaptoethanol, 0.1% gelatin, 20 $\mu$ g/ml of ribonuclease A and 20 units of *Eco*RI (Nippon Gene, Japan). By 0.8% agarose gel electrophoresis of the resulting digest, it was found that this positive clone contains an insert DNA fragment of about 150 bp. The insert DNA was separated and purified using glass powder (Gene Clean™, Bio-l01). About 20 ng of the recovered DNA fragment and about 100 ng of pUC19 vector which has been digested with *Eco*RI were added to the mixture of liquid A 20  $\mu$ l and liquid B 4  $\mu$ l from the DNA ligation kit (Takara Shuzo, Japan), and the resulting mixture was then incubated at 16°C for 15 hours to obtain a recombinant plasmid in which both DNA fragments were linked together. Using 10  $\mu$ l of this reaction mixture, transformation of *E. coli* strain TGI was carried out by the Mandel's method (Mandel, M. and Higa, A., *J. Mol. Biol.*, vol.53, p.154, 1970). The transformant thus obtained was cultured overnight at 37°C in 100 ml of LB medium supplemented with 25  $\mu$ g/ml of ampicillin, and a plasmid DNA was purified from the cultured cells by alkaline lysis method (Birboim, H.C. and Doly, J., *Nucleic Acids Res.*, vol.7, p.1513, 1979). 10  $\mu$ g of the plasmid DNA was digested at 37°C for 1 hour in 200  $\mu$ l of the digestion solution consisting of 100mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM mercaptoethanol, 0.1% gelatin and 100 units of *Eco*RI (Nippon Gene, Japan). The digest was extracted with phenol, concentrated by ethanol precipitation and then subjected to 0.8% agarose gel electrophoresis. Thereafter, an insert DNA fragment of about 150 bp was recovered by glass powder technique, for use as a probe in the following PDI cDNA screening.

In order to obtain a clone which contains the full length human PDI cDNA, screening were carried out again from about 50,000 clones of human Liver  $\lambda$ gt11 cDNA library and about 50,000 clones of human placenta  $\lambda$ gt11 cDNA libraries (Clontech). Filters on which phage DNA molecules of the two libraries were fixed were prepared in the same manner as described in the foregoing. In this instance, about 100 ng of the aforementioned 150 bp human PDI cDNA fragment was isotope-labeled using [ $\alpha$ -<sup>32</sup>P] dCTP (>400 Ci/mmol, Amersham) and a nick translation kit (Amersham), and the labeled cDNA fragment was used as a probe in the screening. After soaking the above two filters in the aforementioned prehybridization solution for 1 hours at 60°C, the filters were further soaked in a hybridization solution (prepared by supplementing the prehybridization solution with about 10<sup>6</sup> cpm/ml of the labeled DNA) for 15 hours at 60°C. The resulting filters were washed with 2 x SSC solution at room temperature and then with 0.5 x SSC + 0.1% sodium dodecyl sarcosinate solution at 65°C for 1 hour, followed by their exposure to X-ray films (XAR-5, Kodak) overnight at -80°C. After development of the films, 6 positive signals were found from the liver cDNA library, and 5 positive signals from the placenta cDNA library. By subjecting these clones to second and third screenings, 4 positive clones were isolated from the liver cDNA library, and 3 positive clones from the placenta cDNA library. The *Eco*RI insert DNA fragments of the obtained 7 clones were separately subcloned into an *Eco*RI site of plasmid vector pUC19 in the same manner as described above in order to make restriction maps for the inserts of the 7 clones. As the results, 4 clones obtained from the liver cDNA library and 2 clones from the placenta cDNA library were found to overlap one another. In addition, it was estimated that the full length human PDI cDNA desired is covered by one of the liver-originated clones (pHDPI16) and one of the placenta-originated clones (pHDPI4) based upon the comparison of restriction maps of these clones with that reported by Pihlajaniemi *et al.* DNA base sequences of the two clones were determined using M13 SEQUENCING KIT (Toyobo, Japan.) M13 Sequencing kit (Takara Shuzo) and an automatic DNA sequencer (370A, Applied Biosystems). Comparison of the thus determined sequences with the data reported by Pihlajaniemi *et al.* confirmed that the full length human PDI cDNA which consist of 2454 base

pairs is encoded by these two clones (SEQ ID No:1).

#### Construction of plasmid for human PDI expression in yeast

A plasmid for use in the expression of human PDI in yeast was constructed by the following procedure, using the above two clones, pHPDI6 and pHPDIp4, which encode human PDI cDNA (Fig. 1):

About 1 µg of pHPDI6 DNA prepared by the alkaline lysis method was digested at 327°C for 1 hour in 20 µl of the digestion solution consisting of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM MgCl<sub>2</sub>, 6 mM mercaptoethanol, 0.1% gelatin, 10 units of *EcoRI* (Nippon Gene) and 10 units of *PstI* (Nippon Gene). The resulting digest was subjected to 0.8% agarose gel electrophoresis and then to the glass powder method to separate and purify a DNA fragment of about 490 bp which corresponds to a 5'-end *EcoRI-PstI* fragment of the PDI cDNA. Separately from this, about 1 µg of pHPDIp4 was digested at 37°C for 1 hour in 20 µl of the digestion solution consisting of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 6 mM MgCl<sub>2</sub>, 6 mM mercaptoethanol, 0.1% gelatin, 10 units of *PstI* (Nippon Gene) and 10 units of *BamHI* (Nippon Gene). The resulting digest was treated in the same manner as described above to separate and purify a DNA fragment of about 1.3 kb which corresponds to a 3'-end *PstI-BamHI* fragment of the PDI cDNA. The thus recovered two DNA fragments (about 50 ng for each) were ligated with about 20 ng of plasmid vector pUC119 which has been digested in a linear form which *EcoRI* and *BamHI*, by incubating these DNA samples at 16°C for 15 hours in the mixture of 25 µl of Liquid A and 5 µl of Liquid B of the DNA ligation kit (Takara Shuzo). With 10 µl of the reaction mixture obtained, a component *E. coli* strain MV1190 cell was transformed by the calcium chloride technique. The transformed cell was cultured overnight at 37°C on an X-Gal plate (LB medium containing 1.5% agar further supplemented with 50 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 80 µg/ml of isopropyl-β-D-thiogalactopyranoside and 25 µg/ml of ampicillin) of 90 mm in diameter. White colonies grown on the plate were picked up, plasmid DNAs were prepared from the colonies by the alkaline lysis method, and the DNAs were analysed using restriction enzymes, thereby selecting a transformant which carries a target plasmid. The thus obtained plasmid was named pHPIEB.

Using a plasmid pHPIEB, a *NaeI* cleavage site was introduced into the boundary region between the PDI signal sequence and the PDI sequence itself on the DNA by the method of Kunkel (Kunkel, T.A., *Proc. Natl. Acad. Sci.*, USA, vol.82, p.448, 1985). *E. coli* strain BW313 competent cell was transformed with the pHPIEB DNA by calcium chloride technique. A single colony of the resultant transformant was pre-cultured overnight at 37°C in 2 x YT medium (1.6% Bacto-trypton, 0.5% NaCl and 1% Bacto-Yeast Extract) supplemented with 150 µg/ml of ampicillin. One ml of the pre-culture was inoculated into 50 ml of the 2 x YT medium supplemented with 150 µg/ml of ampicillin, followed by its culture at 37°C. When turbidity (OD<sub>600</sub>) of the medium reached around 0.3, M13K07 phage (m.o.i. = 2) was added to the medium, and the infection was carried out by incubating the mixture at 37°C for 30 minutes without shaking. To this cell suspension was added kanamycin to a final concentration of 70 µg/ml, followed by culturing at 37°C for 20 hours with shaking. The obtained culture was subjected to centrifugation, and the supernatant recovered was mixed with 1/5 volume of a solution containing 2.5 M NaCl and 20% polyethylene glycol #6000. After stirring, the mixture was left for 15 minutes at room temperature. The precipitate obtained by centrifugation was dissolved in 5 ml of the TE buffer (pH 8.0) consisting of 10 mM Tris-HCl and 1 mM EDTA, mixed with an equal volume of neutral phenol with stirring, and then centrifuged to recover an aqueous layer. To the layer was added an equal volume of chloroform with stirring. The mixture was further subjected to centrifugation to recover an aqueous layer. The aqueous layer was then mixed with 1/10 volume of 3 M sodium acetate and 2.5 volume of ethanol. After stirring, the mixture was left for 30 minutes at -80°C, followed by centrifugation in order to recover DNA as precipitate. The DNA was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 100 µl of the TE buffer.

Using the resulting pHPIEB-originated single-stranded DNA containing dU, a desired mutation, i.e., introduction of a *NaeI* site, was carried out in the following manner:

10 pmol of a synthetic oligonucleotide (5'-CGGGGGCGCCGGCGCGC-3', Takara Shuzo) for use in the introduction of a mutation was incubated at 37°C for 15 minutes in 10 µl of the phosphorylation solution which consists of 100 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 7 mM dithiothreitol, 1 mM ATP and 10 units of T4 polynucleotide kinase (Takara Shuzo), followed by heating at 70°C for 10 minutes in order to deactivate the T4 polynucleotide kinase. Separately from this, 0.2 pmol of the above-described pHPIEB-originated single-stranded DNA and 1 µl of an annealing buffer (Site-directed mutagenesis system Mutan™-K, Takara Shuzo) were mixed with sterile water to a final volume of 10 µl. One µl of this solution was mixed with 1 µl of the phosphorylated synthetic oligonucleotide solution obtained above, and the mixture was left at 65°C for 15 minutes and then at 37°C for 15 minutes. Thereafter, a complementary chain synthesis was carried out by mixing the reaction mixture with 25 µl of a chain elongation solution (Site-directed mutagenesis system Mutan™-K, Takara Shuzo), 60 units of *E. coli* DNA ligase (Mutant™-K, Takara Shuzo) and 1 unit of T4 DNA polymerase (Mutant™-K, Takara

Shuzo), and by incubating the resulting mixture at 25°C for 2 hours. The reaction was determined by adding 3 µl of 0.2 M EDTA (pH 8.0) and heating the mixture at 65°C for 5 minutes. 3 µl of the DNA solution obtained was mixed with 30 µl of a suspension of *E. coli* strain BMH71-18mutS competent cell, and the cell suspension was left for 30 minutes in an ice bath, for 45 seconds at 42°C and then for 1 minute in an ice bath. To the cell suspension was then added 300 µl of SOC medium (2% Bacto-trypton, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO<sub>4</sub>, 10 mM MgCl<sub>2</sub> and 20 mM glucose), and the mixture was shaken at 37°C for 1 hour. 10 µl of M13K07 phage was further added thereto, and the mixture was left for 30 minutes at 37°C. After adding 1 ml of 2 x YT medium containing 150 µg/ml of ampicillin and 70 µg/ml of kanamycin to the mixture, the mixture was shaken at 37°C for 20 hours. The resulting culture was centrifuged to recover 20 µl of supernatant which was subsequently mixed with 80 µl of a culture of *E. coli* strain MVI190. After incubation at 37°C for 10 minutes, the resulting mixture was inoculated onto a LB plate supplemented with 150 µg/ml of ampicillin and cultured overnight at 37°C. Among colonies grown on the plate, a transformant carrying a *NaeI* site-introduced plasmid was identified by DNA-sequencing using M13 SEQUENCING KIT (Toyobo). This plasmid was named phPDINae.

2 µg of the phPDINae DNA prepared by the alkaline lysis method was digested at 37°C for 4 hours in 30 µl of the digestion solution consisting of 10 mM Tris-HCl (pH 8.0), 20 mM NaCl, 7 mM MgCl<sub>2</sub>, 7 units of *NaeI* (Nippon Gene) and 10 units of *HindIII* (Takara Shuzo). The resulting digest was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique so as to separate and purify a DNA fragment of about 1.7 kb.

A plasmid, pUC19Sig, containing a DNA fragment which encodes a human serum albumin prepro-sequence and is composed of codons often utilized in yeast was constructed in the following manner (Fig. 1):

One µg of plasmid vector pUC19 DNA was digested at 37°C for 1 hour in 20 µl of the digestion solution which consist of 100 mM Tris-Hcl (pH 7.5), 10 mM MgCl<sub>2</sub>, 50 mM NaCl and 12 units of *EcoRI* (Nippon Gene), followed by heating at 70°C for 5 minutes in order to deactivate the enzyme. To the reaction mixture was added 38 µl of sterile water and 1 unit of bacterial alkaline phosphatase (Takara Shuzo), and the mixture was incubated at 37°C for 1 hour, followed by phenol extraction and ethanol precipitation to recover DNA. The DNA was then incubated overnight at 16°C in 30 µl of the ligation solution which consisted of 66 mM Tris-HCl (pH 7.5), 6.6 mM mgCl<sub>2</sub>, 10 mM dithiothreitol, 0.1 mM ATP and 300 units of T4 DNA ligase (Takara Shuzo), together with an equal molar amount of a *XhoI* linker containing a *XhoI* site and consisting of the following sequence:

5'-AATTCTCGAG

GAGCTCTTAA-5'.

Using 10 µl of this solution, transformation of *E. coli* JM107 competent cell was carried out by the calcium chloride method. The transformed cell was cultured overnight at 37°C on a LB plate supplemented with 50 µg/ml of ampicillin. Plasmid DNAs were prepared by the alkaline lysis method from the colonies on the plate and analysed using restriction enzymes. In this way, a plasmid DNA molecule in which the *XhoI* linker has been inserted into pUC119 *EcoRI* site was selected.

The following four types of oligonucleotides:

(1) 5'-TCGAGAATTCATGAAGTGGGTTACCTTCATCTCTTTGTTGTT-3';

(2) 5'-AACAAAGAACAACAAAGAGATGAAGGTAACCCACTTCATGAATTC-3';

(3) 5'-CTTGTTCTCTTCTGCTTACTCTAGAGGTGTTTTCAGAAGGCCTG-3'; and

(4) 5'-GATCCAGGCCTTCTGAAAACACCTCTAGAGTAAGCAGAAGAG-3'.

were synthesized using an automatic DNA synthesizer (380B, Applied Biosystems).

Each 5'-end of these oligonucleotides was phosphorylated by incubating about 30 pmol of each sample at 37°C for 1 hour in the solution consisting of 50 mM Tris-HCl (pH 7.6), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.2 mM ATP and 6 units of T4 polynucleotide kinase (Takara Shuzo). The oligonucleotide solutions obtained were combined (100 µl in total volume) and annealed by leaving the combined solution for 5 minutes in a water bath of 100°C, followed by cooling down to room temperature. To the solution was then added 600 units of T4 DNA ligase (Takara Shuzo), and the mixture was left overnight at 16°C to ligate these fragments. The double-stranded DNA preparation thus obtained was subjected to phenol extraction in order to remove proteins, and then to

ethanol precipitation to recover the DNA.

One  $\mu\text{g}$  of the above *Xho*I linker-introducing vector plasmid was digested at 37°C for 1 hour in 20  $\mu\text{l}$  of the digestion solution consisting of 100 mM Tris-HCl (pH 7.5), 10 mM  $\text{MgCl}_2$ , 100 mM NaCl, 10 units of *Bam*HI (Nippon Gene) and 12 units of *Xho*I (Takara Shuzo), followed by phenol extraction and ethanol precipitation to recover a DNA fragment. The fragment obtained was incubated overnight at 16°C in 30  $\mu\text{l}$  of the ligation solution which consist of 66 Tris-HCl (pH 7.5), 6.6 mM  $\text{MgCl}_2$ , 10 mM dithiothreitol, 0.1 mM ATP and 300 units of T4 DNA ligase (Takara Shuzo), together with an equal molar amount of the double-stranded DNA fragment obtained by ligating the four oligonucleotides. Using 10  $\mu\text{l}$  of the thus prepared solution, transformation of *E. coli* JM1017 competent cells was carried out by the calcium chloride method. The transformed cells were cultured overnight at 37°C on LB medium containing 50  $\mu\text{g/ml}$  of ampicillin. Plasmid DNAs prepared from colonies on the plate were analysed using restriction enzymes so as to select a transformant containing a desired recombinant plasmid. The obtained plasmid was named pUC119Sig.

A DNA was prepared from plasmid pUC119Sig by the alkaline lysis method. 2  $\mu\text{g}$  of the DNA was digested at 37°C for 4 hours in the digestion solution which consist of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM  $\text{MgCl}_2$ , 8 units of *Stu*I (Nippon Gene) and 10 units of *Hind*III (Takara Shuzo), subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 3.2 kb. The 1.7 kb DNA fragment (about 50 ng) derived from pHPDINae was reacted with the 3.2 kb DNA fragment (about 50 ng) from pUC119Sig at 16°C for 30 minutes in the ligation kit solution of Takara Shuzo (Japan) (a mixture of liquid A 30  $\mu\text{l}$  + Liquid B 6  $\mu\text{l}$ ). Using 10  $\mu\text{l}$  of its reaction mixture, transformation of *E. coli* HB101 competent cells (Takara Shuzo) was carried out by the calcium chloride method. The transformed cells were cultured overnight at 37°C on a LB plate supplemented with 50  $\mu\text{g/ml}$  of ampicillin. Plasmid DNAs were prepared by the alkaline lysis method from colonies grown on the plate and analysed using restriction enzymes select a recombinant plasmid in which the human PDI itself was linked to the downstream side of the human serum albumin prepro-sequence (Fig. 2). The obtained plasmid was named pHPDILyl.

A human PDI expression plasmid was constructed in the following manner, such that the leader sequence modified type PDI can express under the control of a promoter of yeast alcohol dehydrogenase I gene:

7  $\mu\text{l}$  pHPDILyl DNA prepared by the alkaline lysis method was digested at 37°C for 2 hours in 100  $\mu\text{l}$  of the digestion solution which consists of 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM  $\text{MgCl}_2$  and 40 units of *Eco*RI (Nippon Gene). The resulting solution was mixed with an equal volume of a pheno/chloroform mixture (a mixture of saturated phenol with an equal volume of chloroform). After stirring, the mixture was centrifuged to recover an aqueous layer. The phenol/chloroform extraction was repeated, and the aqueous layer obtained was mixed with 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. The mixture was left for 2 hours at -40°C and then subjected to centrifugation. The pellet was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 50  $\mu\text{l}$  of Klenow buffer solution (Deletion Kit for Kilo-Sequence, Takara Shuzo). Thereafter, to the solution obtained was added 4 units of Klenow fragment (Takara Shuzo), and the mixture was incubated at 37°C for 45 minutes to blunt-end the *Eco*RI cleavage site. The thus prepared solution was subjected twice to phenol/chloroform extraction, and the resulting aqueous layer was mixed with 1/10 volume of 3 M sodium solution acetate (pH 5.3) and 2.5 volume of ethanol. The mixture was left for 1 hour at -40°C and then subjected to centrifugation. The pellet was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 50  $\mu\text{l}$  of Klenow buffer solution (Deletion Kit for Kilo-Sequence, Takara Shuzo). Thereafter, to the solution obtained was added 4 units of Klenow fragment (Takara Shuzo), and the mixture was incubated at 37°C for 45 minutes to blunt the *Eco*RI cleavage site. The reaction mixture was then subjected twice to phenol/chloroform extraction, and the resulting aqueous layer was mixed with 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. The mixture was left for 1 hour at -40°C and then centrifuged. The pellet was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 40  $\mu\text{l}$  of the solution consists of 10 mM Tris-HCl (pH 8.0), 60 mM NaCl, 7 mM  $\text{MgCl}_2$  and 10 units of *Bam*HI (Nippon Gene). Thereafter, the DNA solution obtained was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique so as to separate and purify a DNA fragment of about 1.8 kb. Separately from this, 5  $\mu\text{l}$  of pJDB-ADH-HSA-A DNA (Japanese Patent Application Laying-Open (KOKAI) No. 2-117384) prepared by the alkaline lysis method was digested at 37°C for 2 hours in 100  $\mu\text{l}$  of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM  $\text{MgCl}_2$  and 24 units of *Xho*I (Takara Shuzo). The reaction mixture was then subjected to phenol/chloroform extraction, and the aqueous layer separated was mixed with 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. The mixture was left for 2 hours at -40 °C and then centrifuged to recover DNA as precipitate. The DNA precipitate was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 50  $\mu\text{l}$  of Klenow buffer solution (Deletion Kit for Kilo-Sequence, Takara Shuzo). Thereafter, to the solution obtained was added 4 units of Klenow fragment (Takara Shuzo), and the mixture was incubated at 37°C for 45 minutes to blunt the *Xho*I cleavage site. The reaction mixture was then subjected twice to phenol/chloroform extraction, and the aqueous layer separated was mixed with 1/10 volume

3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. The mixture was left for 1 hour at -40°C before centrifugation. The DNA pellet removed was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 40 µl of the solution which consist of 10 mM Tris-HCl (Ph 8.0), 60 mM NaCl, 7mM MgCl<sub>2</sub> and 10 units of *Bam*HI (Nippon Gene). The solution obtained was incubated at 37°C for 75 minutes to digest the DNA.

5 To the reaction mixture was then added 10 µl of 2 x 2 M Tris-HCl (pH 8.0), 110 µl of sterile water and 1 unit of alkaline phosphatase from *E. coli* strain C75 (Takara Shuzo), and the mixture was incubated at 60°C for 1 hour in order to carry out a 5'-end dephosphorylation of the restriction enzyme-formed cleavage site. To the reaction mixture was added 1/10 volume of 3 M sodium acetate (pH 5.3) and 2.5 volume of ethanol. The mixture obtained was left for 1 hour at -40°C before centrifugation. The DNA pellet separated was dried under a reduced pressure

10 and then dissolved in 20 µl of the TE buffer. Thereafter, the prepared DNA solution was subjected to 0.8% agarose gel electrophoresis and then treated by the powder gas technique so as to separate and purify a DNA fragment of about 8 kb. The thus obtained pHpDILyl1-originated 1.8 kb DNA fragment (about 50 ng) and pJDB-ADH-HSA-A-originated 8 kb DNA fragment (about 50 ng) were incubated at 16°C for 2.5 hours in the DNA ligation kit solution of Takara Shuzo (a mixture of Liquid A 30 µl + Liquid B 6 µl) in order to ligate the two DNA

15 fragments. Using 10 µl of the prepared DNA solution, transformation of *E. coli* strain C600 was carried out by the calcium chloride technique. The transformed cells were cultured overnight at 37°C on a LB plate supplemented with 50 µg/ml of ampicillin. Plasmid DNAs were prepared by the alkaline lysis method from colonies grown on the plate and analysed using restriction enzymes in order to select a transformant carrying a plasmid in which the leader sequence modified type PDI sequence was linked to the downstream side of the alcohol dehydrogenase I promoter. The constructed PDI expression plasmid was named pAHhPDIyl. As the results

20 of the plasmid construction, the N-terminal amino acid of the mature PDI protein was changed from Asp to Gly.

A control plasmid for use in experiments of the human PDI expression was constructed in the following manner:

5 µl of pJDB-ADH-HSA-A DNA prepared by the alkaline lysis method was digested at 37°C for 2 hours

25 in 100 µl of the digestion solution which consists of 10 mM Tris-HCl, 100 mM NaCl, 7 mM MgCl<sub>2</sub>, 24 units of *Xho*I (Takara Shuzo) and 29 units of *Bam*HI (Nippon Gene). The reaction mixture obtained was subjected twice to phenol/chloroform extraction, and to the aqueous layer was added 1/10 vol of 3 M sodium acetate (pH 5.3) and 2.5 vol of ethanol. The mixture obtained was then left for 2 hours at -40°C and then centrifuged to recover DNA as pellet. The DNA pellet was washed with 70% ethanol, dried under a reduced pressure and then dissolved in 50 µl of Klenow buffer solution (Deletion Kit for Kilo-Sequence, Takara Shuzo). Thereafter, to the obtained solution was added a 4 units of Klenow fragment (Takara Shuzo), and the mixture was incubated at 37°C

30 for 45 minutes to blunt the *Xho*I and *Bam*HI cleavage sites. The reaction mixture was then subjected twice to phenol/chloroform extraction, and the aqueous layer separated was mixed with 1/10 vol of 3 M sodium acetate (pH 5.3) and 2.5 vol of ethanol. The mixture was left for 1 hour at -40°C before centrifugation. The DNA pellet removed was dried under a reduced pressure and dissolved in 20 µl of the TE buffer. Thereafter, the DNA solution was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 8 kb. The thus obtained DNA fragment (about 50 ng) was mixed

35 with the mixture of liquid A 30 µl + Liquid B 6 µl from the DNA ligation kit (Takara Shuzo), and incubated overnight at 16°C so as to cyclize it by self-ligation. Using 10 µl of the prepared DNA solution, transformation of *E. coli* 101 competent cells (Takara Shuzo) was carried out by the calcium chloride technique. The transformed cells were cultured overnight at 37°C on a LB plate supplemented with 50 µg/ml of ampicillin. Plasmid DNAs were prepared by the alkaline lysis technique from the colonies grown on the plate, and analyzed using restriction enzymes in order to select a desired control plasmid. The constructed plasmid was named pAH

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#### 45 Expression of human PDI yeast

Using the human PDI expression plasmid pAHhPDIyl constructed above, an expression of human PDI yeast was carried out in the following manner:

A single colony of yeast strain AH22 obtained by culturing it on a YPD plate (2% Bacto-pepton, 1% yeast extract, 2% glucose and 1.5% agar) was inoculated into 5 ml of a YPD medium (2% Bacto-pepton 1% yeast extract and 2% glucose) and cultured at 30°C for 24 hours with shaking. This pre-culture (0.9 ml) was inoculated

50 into 45 ml of the YPD medium and cultured at 30°C with shaking. When turbidity at OD<sub>600</sub> reached about 0.5, the main culture was subjected to a low speed centrifugation to recover yeast cells as precipitate. The cells removed were suspended in 3 ml of 0.2 M LiSCN, and the cell suspension (1 ml) was centrifuged to recover

55 the cells. To the cells were subsequently added 46 µl of 50% PEG #4000, 10 µl of LiSCN and 10 µl of a pAHhPDIyl DNA solution (27 µg as DNA) prepared by the alkaline lysis method. After mixing them by pipetting the mixture was left overnight at 30°C, followed by its suspension in 1 ml of sterile water. The suspension was then centrifuged to recover cells as pellet. The pellet was resuspended in 100 µl of sterile water and cultured at 30°C



after the inoculation of its suspension onto a SD(-Leu) plate (SD(-Leu) medium (0.67% Bacto-nitrogen base, 2% glucose, 20 mg/l of adenine, 20 mg/l of uracil, 20 mg/l of tryptophan, 20 mg/l of histidine, 20 mg/l of arginine, 20 mg/l of methionine, 30 mg/l of tyrosine, 30 mg/l of isoleucine, 30 mg/l of lysine, 50 mg/l of phenylalanine, 100 mg/l of aspartic acid, 100 mg/l glutamic acid, 150 mg/l of vaseline, 200 mg/l of threonine and 375 mg/l of serine (amino acids from Wako Pure Chemical Industries, Japan)) + 1.5% agar). A transformant from the 5-days culture was inoculated into 5 ml of SD (-Leu) medium and cultured at 30°C for 2 days with shaking. 100 µl of the obtained pre-culture was then inoculated into 5 ml of the YPD medium and cultured at 30°C for 24 hours with shaking. 1.5 ml of the resulting main culture was centrifuged to recover 500 µl of supernatant which was subsequently mixed with the equal volume of ethanol and then left for 1 hour in an ice bath. The mixture was centrifuged so as to recover products secreted out of the yeast cells as a pellet which was then dried under a reduced pressure. The pellet was dissolved in 10 µl of a sample buffer for SDS-PAGE (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.01% Bromophenol Blue). After boiling for 5 minutes, the treated sample was subjected to electrophoresis on SDS/PAGE plate 10/20 (Daiichi Kagaku Yakuhin, Japan). The resulting gel was stained with a staining solution (0.15% Coomassie Brilliant Blue, 10% acetic acid and 40% methanol) and then soaked in a decoloring solution (10% acetic acid and 40% methanol) to visualize an expressed product. In this instance, a control sample obtained by the same procedure, except that pAHhP-DILyl was replaced by the aforementioned control plasmid pAH, was run at the same time during the electrophoresis. As standard molecular weight markers, phosphorylase b (molecular weight, 94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α-lactalbumin (14,000) were used (Fig. 3). As the results, an expression product having a molecular weight of about 55 K was found. Since this molecular weight coincided with that of the mature PDI protein, it was assumed that a desired human PDI was expressed and secreted. Next, a large-scale culture was carried out in the following manner in order to examine chemical properties of the expressed and secreted protein:

A single colony of the pAHhPDIyl-carrying yeast strain AH22 was inoculated into 80 ml of the SD (-Leu) medium and cultured at 30°C for 2 days with shaking. The obtained pre-culture was then inoculated into 4 liters of a YPD-phosphate medium (YPD medium, 6 g/l of Na<sub>2</sub>HPO<sub>4</sub>, and 3 g/l of KH<sub>2</sub>PO<sub>4</sub>, pH 7.0) and cultured at 30°C for 24 hours with shaking. The resulting main culture was centrifuged to removed the supernatant which was used for the purification of the secreted expression product.

#### Isolation of recombinant human PDI from the culture and its characterization

The culture (4 liters) obtained by culturing the recombinant yeast was concentrated to 1/40 (final volume, 100 ml) using a Millipore-Millitan ultrafiltration apparatus (nominal molecular weight, 30,000 cut-off), and then subjected to TSK-gel Phenyl-5PW hydrophobic column chromatography so as to isolate human PDI. The elution was carried out in 10 mM borate-10 KCl buffer (pH 8.0) containing 0.05% NaN<sub>3</sub>, with a linear gradient from 0.85 M to 0 M of ammonium sulfate over 125 minutes. The flow rate was 2 ml/min. The result is shown in Fig. 4. In Fig. 5 the result of SDS-electrophoresis of the isolated human PDI is illustrated. As shown in the figures, the human PDI was purified almost homogeneously by the hydrophobic column chromatography without a loss of its activity. Any UV-absorbing substance in the YPD medium could be removed markedly efficiently by the chromatography.

#### PDI assay

PDI assay was carried out by measuring its effect to enhance the refolding of scrambled ribonuclease A (RNase A) which has been prepared by reduction, denaturation and re-oxidation steps. Refolding degree of the scrambled RNase A was determined by measuring a degree of the restoration of its enzyme activity. The following describes the assay procedure illustratively:

##### (A) Preparation of scrambled RNase A:

120 mg of RNase A was dissolved in 3 ml of 0.1 M Tris-HCl buffer (pH 8.6) containing 6 M guanidine hydrochloride and 0.15 M dithiothreitol, and then reduced under nitrogen atmosphere at room temperature for 15 hours. The reduced product was applied to a Sephadex G-25 column (15 mmØ x 38 cm) equilibrated with 0.01 N HCL, thereby removing the reducing agent. To the desalting product was added guanidine hydrochloride to a final concentration of 6 M. After adjusting its pH value to 9.0 with Tris, the mixture was subjected to an exchange reaction of a S-S bond(s) in the dark at 4°C for 14 days. The thus prepared sample was stored at -80°C for use as the scramble RNase A.

(B) PDI assay:

10  $\mu$ l of 1 dithiothreitol is added to 20 ml of 55 mM phosphate buffer (pH 7.5) in which any dissolving air was replaced with nitrogen gas. 10  $\mu$ l of this solution is added to 420  $\mu$ l of 55 mM phosphate buffer (pH 7.5) mixed with 20  $\mu$ l of an enzyme sample, and the mixture is left for 5.5 minutes at 30°C. To this solution is added 50  $\mu$ l of the scrambled RNase A solution prepared above, followed by the enzymatic reaction at 30°C for 15:5 minutes. Separately from this, 1.945 ml of degassed 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM  $MgCl_2$ , 25 mM KCl and 50  $\mu$ l of a yeast RNA solution (dissolved in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA; adjusted to such a concentration that absorbance at 280 nm become 80) is placed into a quartz cell (1cm x 1cm). With stirring, a temperature of the resulting mixture is maintained at 45°C. In this instance, absorbance at 260 nm should not be changed by the treatment. Therefore, 5  $\mu$ l of the dithiothreitol-treated scrambled RNase A solution is added to the buffer in the quartz cell and, with stirring, a change in the absorbance of the reaction mixture at 260 nm are measured over 2 minutes at 0.2 minute intervals. The PDI activity is calculated from an initial velocity of the changing rate of absorbance at 260 nm.

Example 2Transformation of yeast strain HIS23 with human PDI expression plasmid pAHhPDILyI

Using the aforementioned human PDI expression plasmid pAHhPDILyI, transformation of the HSA-producing yeast strain HIS23 (Japanese Patent Application No. 2-57885 filed by the present applicant, Bikoken-Kin-Ki No. 11351 (FERM P-1138)) was carried out in the following manner.

A single colony of the HSA-expressing yeast strain HIS23 obtained by culturing it on a YPD plate (2% Bacto-trypton, 1% Bacto-yeast extracts, 2% glucose and 1.5% agar) was inoculated into 5 ml of a YPD medium (2% Bacto-trypton, 1% yeast extract and 2% glucose) and cultured at 30°C for 24 hours with shaking. One ml of the obtained pre-culture was inoculated into 50 ml of the YPD medium and cultured at 30°C with shaking. When turbidity at  $OD_{600}$  reached about 0.5, the main culture was subjected to a low speed centrifugation to recover the yeast cells as pellet. To the pellet were added 46  $\mu$ l of 50% polyethylene glycol #4000, 10  $\mu$ l of LiSCN and 10  $\mu$ l of the human PDI expression plasmid pAHhPDILyI DNA solution (about 20  $\mu$ g as DNA) prepared by the alkaline lysis method (Birboim, H.C. and Doly, J., *Nucleic Acid Res.*, vol.7 p.1513 1979). After mixing them by pipetting, the mixture was left overnight at 30°C. The resulting mixture was suspended in 1 ml of sterile water and centrifuged to recover cells at pellet. The pellet was suspended in 100  $\mu$ l of sterile water and cultured at 30°C after inoculating the cell suspension onto a SD (-His,-Leu) plate (0.67% Bacto-nitrogen base, 2% glucose, 20 mg/l of adenine, 20 mg/l of uracil, 20 mg/l of tryptophan, 20 mg/l of arginine, 20 mg/l of methionine, 30 mg/l of tyrosine, 30 mg/l of isoleucine, 30 mg/l of lysine, 50 mg/l of phenylalanine, 100 mg/l of aspartic acid, 100 mg/l of glutamic acid, 150 mg/l of valine, 200 mg/l of threonine and 375 mg/l of serine (amino acids from Wako Pure Chemical Industries)) + 1.5% agar). A transformant was obtained as a colony grown on the plate on the day 5 after the culture.

Expression of PDI on the obtained transformant (pAHhPDILyI)/HIS23) was examined in the following manner:

In this instance, a transformant (pAH,HIS23) obtained using the plasmid pAH which has been prepared by removing the PDI cDNA moiety from pAHhPDILyI was used as a control. The single colony grown on the plate was inoculated into 5 ml of the SD (-His, -Leu) medium and cultured at 30°C for 2 days with shaking. 100  $\mu$ l of the pre-culture was then inoculated into 5 ml of the YPD medium and cultured at 30°C for 24 hours with shaking. 1.5 ml of the resulting main culture was centrifuged to recover 500  $\mu$ l of supernatant which was subsequently mixed with the equal volume of ethanol and then left for 1 hour in an ice bath. The mixture was centrifuged to recover products secreted out of the yeast cells as a precipitate which was then dried under a reduced pressure using an evaporator. The precipitate was dissolved in 10  $\mu$ l of a sample buffer for SDS-PAGE (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5%  $\beta$ -mercaptoethanol, 0.005% Bromophenol Blue and 20% glycerol). After boiling for 5 minutes, the sample was subjected to electrophoresis on SDS-PAGE Plate 4/20-1010 (Daiichi Kagaku Yakuhin). The resulting gel was stained with a staining solution (0.15% coomassie Brilliant Blue, 10% acetic acid and 40% methanol) and then soaked in a decoloring solution (10% acetic acid and 40% methanol) to visualize an expressed product. In this instance, phosphorylase b (molecular weight, 94,000 daltons), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and  $\alpha$ -lactalbumin (14,000) were used as standard molecular weight markers (Fig. 6). As the results, the secretion of an expressed PDI having a molecular weight of about 55,000 daltons was detected in the yeast strain HIS23 transformed with pAHhPDILyI.

Effect of human PDI on the expression secretion of HSA

Using the above described co-expression system of HSA and PDI in a yeast cell, effect of human PDI on the expression and secretion of HSA were examined in the following manner:

- 5 Five single colonies were isolated from strain pAH/HIS23 which has been obtained by transforming the yeast strain HIS23 with the control plasmid pAH, and other 5 single colonies were isolated from strain pAHhP-DILy/HIS23 obtained by transforming the HIS23 with the human PDI expression plasmid pAHhPDILy. Each of the thus isolated single colonies was inoculated into 5 ml of the SD (-His, -Leu) medium and cultured at 30°C for 24 hours. Each of the pre-cultures (100 ml) was inoculated into 5 ml of the YPD medium and cultured at 10 30°C for 24 hours with shaking. From the main cultures obtained, samples for SDS-PAGE were prepared in accordance with the aforementioned procedure. Results the SD-PAGE are shown in Fig. 7. Using the gels subjected to the SDS-PAGE, the amount of secreted HSA from each strain was determined using a densitometer (IMAGE ANALYSIS SYSTEM, TEFCO, Japan) in order to examine effects of the co-expression of PDI on the secreted amount expression HSA (Fig. 8). As shown in the figure, the strain pAH/HIS23 and pAHhPDILy secreted HSA in average amounts of 0.93 mg/l; and 1.50 mg/l, respectively. In other words, secretion of HSA was 15 increased by about 60% in average due to the co-expression of PDI in the yeast strain HIS23.

Example 320 Cloning of yeast ERD2 gene

Cloning of yeast ERD2 gene was carried out through the polymerase chain reaction (PCR) technique (Mullis, K.B. and Faloona, F., *Meth. Enzymol.*, vol.155, p.335, 1987) as follows:

- A single colony of yeast strain S288C was inoculated into 2 ml of YPD medium (2% Bacto-trypton, 1% 25 Bacto-yeast extracts and 2% glucose) and cultured at 30°C for 24 hours. Yeast cells recovered by centrifugation from the culture were washed with 1 ml of a sorbitol solution (1 M sorbitol and 50 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>; pH 6.85) and then subjected again to centrifugation. The cells collected were suspended in 1 ml of the sorbitol solution, and the cell suspension was mixed with 40 µl of Zymolyase solution (10 mg/ml) and 1 µl of β-mercaptoethanol, and maintained at 37°C for 30 minutes. Next, cells recovered by centrifugation were suspended in 200 µl of 30 50 mM Tris-HCl (pH 7.5) containing 20 mM EDTA, and to the cell suspension was added 200 µl of an NDS buffer solution (0.5 M EDTA, 10 mM Tris-HCl (pH 7.5), 1% sodium dodecyl sarcosinate, 7.5% β-mercaptoethanol and 1 mg/ml of Pronase K). After incubation at 50°C for 1 hour, the mixture was extracted with a phenol/chloroform solution, and to the aqueous layer separated was added 1/10 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol of ethanol. After cooling at -80°C for 20 minutes, the precipitate obtained by ethanol precipitation was recovered by centrifugation. The precipitate was dissolved in 400 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 35 mM EDTA), and the solution was mixed with 240 µl of 20% PEG solution (20% polyethylene glycol and 2.5 M NaCl). After cooling for 1 hour in an ice bath, the precipitate was recovered by centrifugation which was subsequently washed with 70% ethanol and dissolved in 400 µl of the TE buffer. The solution obtained was extracted twice with phenolchloroform, and a genomic DNA of the yeast strain S288C was recovered by ethanol 40 precipitation of the separated aqueous layer.

Using the thus obtained genomic DNA as a template, cloning of the yeast ERD2 gene was carried out.

- A reaction mixture for PCR reaction was prepared by mixing together 10 µl of S288C genomic DNA (0.02 µg), 5 µg of primer I (0.25 µg), 5 µl of primer II (0.25 µg), 0.5 µg of TaqI polymerase (Gene Amp™ DNA Amplification Reagent Kit, Perkin Elmer Cetus), 10 µl of the reaction buffer concentrated to 1/10 (the same kit just 45 described), dNTP mixture (1.25 mM for each, the same kit just described) and 53.5 µl of sterile water. A PCR reaction was carried out in the mixture using a DNA Thermal Cycler (Perkin Elmer Cetus). The reaction conditions employed are: denaturation of DNA, 94°C for 1 minute; annealing, 50°C for 2 minutes; and polymerase chain elongation reaction, 72°C for 3 minutes. After 30 cycles of the reaction steps, the final reaction was carried out at 72°C for 7 minutes. The primers I and II have the following sequences:

50 **primer I: 5'-TTTTTCTCGAGTAAGCAATGAATCCGTT-3'; and**

**primer II: 5'-AAAAAGGATCCTGCGAACACTATTTAAA-3'.**

55 The reaction mixture obtained was extracted with phenol/chloroform, and the aqueous layer separated was subjected to ethanol precipitation to recover DNA. The DNA was incubated at 37°C for 2 hours in 20 µl of the digestion solution which consist of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub>, 5 units of XhoI (Takara

Shuzo) and 5 units of *Bam*HI (Takara Shuzo). After the reaction, the digest was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique (Gene Clean™, Bio-101) to separate and purify a DNA fragment of about 0.7 kb. About 50 ng of the recovered DNA fragment and about 20 ng of a plasmid vector BluescriptII SK+ which has been digested with *Xho*I and *Bam*HI were mixed with a ligation solution (Liquid A 30 µl + Liquid B 6 µl from the DNA ligation kit, Takara Shuzo), and the mixture was incubated at 16°C for 2 hours so as to ligate the DNA fragment with the plasmid vector. Using the obtained recombinant plasmid (named pYERD2, Fig. 9), transformation of *E. coli* strain XLI-Blue was carried out.

The DNA fragment thus subcloned was further subcloned into small fragments before its DNA sequencing. As the results, it was confirmed that the DNA fragment contained the yeast ERD2 gene. The base sequence and deduced amino acid sequence coincided with those reported by Semenza *et al.* (Semenza, J.C., Hardwick, K.G., Dean, N. and Pelham, H.R.B., *Cell*, vol.61, p.1349, 1990), except that the reported codon for the Leu of position 52 was "TTG" while that of the clone of this invention was "TTA".

#### Construction of vector for use in the integration of ERD2 expression unit into yeast chromosome TRPI site

A vector for use in the integration of ERD2 expression unit into yeast chromosome was constructed using the aforementioned plasmid pYERD2 by the following procedure (Figs. 9 to 11) :

0.5 µg of the plasmid vector pRS304 DNA (Sikorski, R.S. and Hieter, P., *Genetics*, vol.122, p.1, 1989) prepared by the alkaline lysis method (Birboim, H.C. and Doly, J., *Nucleic Acids Res.*, vol.7, p.1513, 1979) was digested at 37°C for 2 hours in 30 µl of a digestion solution which consists of 10 mM Tris-HCl (pH, 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub>, 5 units of *Xho*I (Takara Shuzo) and 5 units of *Bam*HI (Takara Shuzo). The resulting digest was subjected to 0.7% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 4.3 kb. Separately from this, 0.5 µg of the ADH I transcription terminator cassette vector pUC-ATE DNA (Japanese Patent Application Laying-Open (KOKAI) No. 2-117384 filed by the present applicant) was digested at 37°C for 2 hours in 30 µl of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 7 mM MgCl<sub>2</sub>, 5 units of *Sa*I (Takara Shuzo) and 5 units of *Bam*HI (Takara Shuzo). the resulting digest was subjected to 1% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 0.4 kb. The thus recovered DNA fragments (about 50 ng for each) were mixed with a ligation solution (Liquid A 30 µl + Liquid B 6 µl from the DNA ligation kit, Takara Shuzo), and incubated at 16°C for 2 hours to ligate and cyclize the DNA fragments. With the obtained recombinant plasmid (named pRS304-ATE), the *E. coli* strain XL1-Blue was transformed.

Next, 0.5 µg of the HSA expression vector pRG-UAS1-N7-TLY1-305 DNA (Japanese Patent Application No. 3-188794 filed by the present applicant) was digested at 37°C for 2 hours in 30 µl of the digestion solution which consist of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub>, 10 units of *Not*I (Toyobo) and 5 units of *Bam*HI (Takara Shuzo). The resulting digest was subjected to 0.7% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 4 kb. Separately from this, 0.5 µg of the pRS304-ATE DNA was digested at 37°C for 2 hours in 30 µl of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub>, 10 units of *Not*I (Toyobo) and 5 units of *Bam*HI (Takara Shuzo). The resulting digest was subjected to 0.7% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 5 kb. The thus recovered DNA fragments (about 50 ng for each) were mixed with a ligation solution (Liquid A 30 µl + Liquid B 6 µl from the DNA ligation kit, Takara Shuzo), and incubated at 16°C for 2 hours to ligate and cyclize the DNA fragments. Using the obtained recombinant plasmid (named pRG-UAS1-N7-TLY1-304), transformation of the *E. coli* strain XLI-Blue was carried out.

0.5 µg of the aforementioned plasmid pYERD2 DNA prepared by the alkaline lysis method was digested at 37°C for 2 hours in 30 µl of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub>, 5 units of *Xho*I (Takara Shuzo) and 5 units of *Bam*HI (Takara Shuzo). The resulting digest was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 0.7 kb. Separately from this, 0.5 µg of the DNA prepared by the alkaline lysis method from the aforementioned human HSA integration vector pRG-U1-N7-TLY-304 was digested at 37°C for 2 hours in 30 µl of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub>, 5 units of *Xho*I (Takara Shuzo) and 5 units of *Bam*HI (Takara Shuzo). Following 0.8% agarose gel electrophoresis of the resultant digest, a DNA fragment of about 6.3 kb was purified by the glass powder technique. The thus recovered DNA fragments (about 50 ng for each) were mixed with the ligation solution (Liquid A 30 µl + Liquid B 6 µl, Takara Shuzo), and incubated at 16°C for 2 hours to ligate the DNA fragments. With the obtained recombinant plasmid (named pIVTRPGAPYERD2), the *E. coli* strain HBI01 was transformed.

One µg of the pIVTRPGAPYERD2 DNA prepared by the alkaline lysis method was digested at 37°C for 4 hours in 30 µl of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub>,

5 units of *Xho*I (Takara Shuzo) and 5 units of *Hind*III (Takara Shuzo). The resulting digest was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 6.8 kb. Separately from this, 1 µg of DNA prepared by the alkaline lysis method from the HSA expression vector pJDB-ADH-HSA-A (Japanese Patent Application Laying-Open (KOKAI) No. 2-117384) was digested at 37°C for 4 hours in 30 µl of the digestion solution which consist of 10 mM Tris-HCL (pH 8.0), 100 mM NaCL, 7 mM MgCl<sub>2</sub>, 5 units of *Xho*I (Takara Shuzo) and 5 units of *Hind*III (Takara Shuzo). The resulting digest was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment (yeast alcohol dehydrogenase I promoter) of about 1.4 kb. The thus recovered DNA fragments (about 50 ng for each) were mixed with the ligation solution (Liquid A 30 µl + Liquid B 6 µl from the DAN ligation kit, Takara Shuzo), and then inoculated at 16° for 2 hours so as to ligate the DNA fragments. Using the thus obtained recombinant plasmid (named pIVTRPAPHYERD2), transformation of the *E. coli* strain HB101 was carried out.

#### Preparation of strain SN35A

The HSA expression vector pRG-UASI-N7-TLY1-305 (Japanese Patent Application No. 3-188794) was digested with *Hind*III and *Xho*I, and the digest was subjected to 0.7% agarose gel electrophoresis to separate a DNA fragment of about 10 kb which was subsequently purified using Gene Clean. Separately from this, the plasmid pSNAD3AX (Japanese Patent Application No. 3-136657 filed by the present applicant) was digested with *Hind*III and *Xho*I, and the digest was subjected to 1% agarose gel electrophoresis to separate a DNA fragment of about 0.5 kb which was subsequently purified using Gene Clean. The plasmid pSNAD3AX contains a promoter which is composed of a hybrid UAS consisting of a UAS fragment from *E. coli* chromosome and ADH II UAS an ADH I transcription initiation region. The 0.5 kb fragment was then ligated with the above 10 kb fragment using T4 DNA ligase to give a cyclized product, and the aforementioned *E. coli* strain XL1-Blue was transformed with the product to prepare a transformant carrying the HSA expression vector pRG-SNAD3AX-TLY1-305.

On the other hand, the plasmid vector pRS303 (Sikorski and Hieter, 1989) was digested with *Bam*HI and *Xho*I, and the digest was subjected to 0.7% agarose gel electrophoresis to separate a DNA fragment to about 4.3 kb which was subsequently purified using Gene Clean. Separately from this, the ADH I transcription terminator cassette vector pUC-ATE (Japanese Patent Application Laying-Open (KOKAI) No. 2-117384) was digested with *Bam*HI and *Sal*I, and the digest was subjected to 1% agarose gel electrophoresis to separate a DNA fragment of about 0.4 kb which was further purified using Gene Clean. The 0.4 kb fragment was ligated with the above 4.3 kb fragment using T4 DNA ligase to give a cyclized product, and the *E. coli* strain XLI-Blue was transformed with the product to prepare a transformant carrying the plasmid pRS303-ATE.

Next, the HSA expression vector pRG-SNAD3AX-TLY1-305 obtained above was digested with *Not*I and *Bam*HI, and the digest was subjected to 0.7% agarose gel electrophoresis to isolate a DNA fragment of about 4 kb which was subsequently purified using Gene Clean. Separately from this, the plasmid pRS303-ATE obtained above was digested with *Not*I and *Bam*I, and the digest was subjected to 0.7% agarose gel electrophoresis to separate a DNA fragment of about 4 kb which was subsequently purified Gene Clean. This fragment was ligated with the above 4 kb fragment from the pRG-SNAD3AX-TLY1-305 using T4 DNA ligase to give a cyclized product, and the *E. coli* strain XLI-Blue was transformed with the product to prepare a transformant carrying the HSA expression vector pRG-SNAD3AX-TLY1-305.

10 µg of the thus-obtained HSA expression vector pRG-SNAD3AX-TLY1-305 was digested with *Sp*I in 20 µl of a digestion buffer, followed by heating of the resulting reaction mixture at 65°C for 10 minutes in order to deactivate the enzyme. Separately from this, the yeast strain YY35A (FERM P-12480) was cultured overnight in a YPD medium (1% yeast extract, 2% peptone and 2% glucose). 0.1 ml of the obtained pre-culture was inoculated into 5 ml of the YPD medium, and cultured until turbidity at OD<sub>600</sub> reached 1.0. The main culture obtained was subjected to centrifugation to recover cells which were subsequently washed with 0.5 ml of 0.1 M lithium acetate solution containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The cells suspended were again collected by centrifugation, resuspended in 70 µl of the 0.1 M lithium acetate solution, and then incubated at 30°C for 1 hour.

The thus treated cell suspension was mixed with 20 µl of the just described heat-treated reaction mixture, and the mixture was incubated at 30°C for 30 minutes. To the mixture was added 500 µl of 0.1 M lithium acetate solution containing 40% polyethylene glycol (average molecular weight, 4000), followed by fully mixing using a Pipette-man. After incubation at 30°C for 45 minutes, the cell suspension was warmed at 42°C for 5 minutes, mixed with 500 µl of sterile water, and then centrifuged to recover the cells. The obtained cells were resuspended in 100 µl of sterile water and spreaded over the SD (-His) agar medium which consist of 20 µg/ml of adenine sulfate, 20 µg/ml of arginine hydrochloride, 20 µg/ml of methionine, 20 µg/ml of thryptophan, 20 µg/ml

of uracil, 30 µg/ml of isoleucine, 30 µg/ml of lysine hydrochloride, 30 µg/ml of tyrosine, 50 µg/ml of phenylalanine, 60 µg/ml of leucine, 150 µg/ml of valine, 0.67 amino acid-free Yeast Nitrogen Base, 2% glucose and 2% agar. After culturing the cells at 30°C for 5 days, One of the colonies grown on the plate was picked up and cultured again on the SD (-His) agar medium in the same manner to obtain a purified HSA-highly producing strain SN35A.

#### Preparation of yeast strain SN35A-1PU

##### (A) Construction of human PDI expression unit using TDH3 promoter:

4 µg of the plasmid pHPDILyl DNA (Japanese Patent Application No. 3-114074 filed by the present applicant) prepared by the alkaline lysis technique was digested at 37°C for 2 hours in 100 µl of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 60 mM NaCl, 7 mM MgCl<sub>2</sub> and 20 units of *EcoRI* (Nippon Gene). The reaction mixture was subjected to phenol/chloroform extraction and then to ethanol precipitation to recover the digested DNA. The DNA precipitate was dissolved in 50 µl of Klenow buffer solution (Deletion Kit for Kilo-Sequence, Takara Shuzo), mixed with 4 units of Klenow fragment (Takara Shuzo), and then incubated at 37°C for 45 minutes to blunt the *EcoRI* cleavage site. This reaction mixture was subjected to phenol/chloroform and then to ethanol precipitation to recover DNA. The DNA obtained was dissolved in 30 µl of a digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub> and 10 units of *BamHI* (Nippon Gene), and then incubated at 37°C for 2 hours so as to digest the DNA. Thereafter, the resulting digest was subjected to 0.8% agarose gel electrophoresis to separate a DNA fragment of about 1.8 kb which was further purified using Gene Clean.

Separately from this, 3 µg of the plasmid pRG-UASI-N7-TLYI-305 DNA prepared by the alkaline lysis technique was digested at 37°C for 2 hours in 100 µl of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub> and 24 units of *XhoI* (Takara Shuzo). The resulting digest was subjected to phenol/chloroform extraction and then to ethanol precipitation to recover the digested DNA. The DNA precipitate was dissolved in 50 µl of the Klenow buffer solution. The solution obtained was mixed with 4 units of the Klenow fragment and incubated at 37°C for 45 minutes to blunt the *XhoI* cleavage site. The reaction mixture was then subjected to phenol/chloroform extraction and to ethanol precipitation so as to recover DNA. The DNA was dissolved in 40 µl of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub> and 10 units of *BamHI* (Nippon Gene), and then incubated at 37°C for 2 hours to digest the DNA. After ethanol precipitation, the digested DNA precipitate was subjected to 0.8% agarose gel electrophoresis to separate a DNA fragment of about 8 kb which was subsequently purified using Gene Clean.

The thus recovered 1.8 kb DNA fragment from pHPDILyl (about 50 ng) and 8 kb DNA fragment from pRG-UASI-N7-TLYI-305 (about 50 ng) were mixed with the ligation solution (Liquid A 30 µl + Liquid B 6 µl, takara Shuzo), and then incubated at 16°C for 1 hour to ligate the DNA fragments. Using 10 µl of the resulting DNA solution, transformation of the *E. Coli* strain HB101 was carried out by the calcium chloride technique (Mandel, M. and Higa, A., *J. Mol. Biol.*, vol.53, p.154, 1970). Thereafter, a transformant containing the desired plasmid (named pIVLEUGAPhPDILyl) was selected by restriction analysis.

2 µg of the pIVLEUGAPhPDILyl plasmid DNA prepared by the alkaline lysis technique was digested at 37°C for 2 hours in 40 µl of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 60 mM NaCl, 7 mM MgCl<sub>2</sub>, 10 units of *BamHI* (Nippon Gene) and 10 units of *HindIII* (Nippon Gene). The resulting digest was subjected to 0.8% agarose gel electrophoresis to separate a DNA fragment of about 2.5 kb which was subsequently purified using Gene Clean. Separately from this, 2 µg of the plasmid pJDB-ADH-HSA-A DNA (Japanese Patent Application Laying-Open (KOKAI) No. 2-117384) prepared by the alkaline lysis technique was digested under the similar conditions to those in the digestion of pIVLEUGAPhPDILyl DNA. The resulting digest was subjected to 0.8% agarose gel electrophoresis to separate a DNA fragment of about 8.5 kb which was subsequently purified using Gene Clean. The two DNA fragments (about 50 ng for each) were mixed with the ligation solution (Liquid A 30 µl + Liquid B 6 µl from the DNA ligation kit, Takara Shuzo), and then incubated at 16°C for 1 hour so as to ligate the DNA fragments. Using 10 µl of the obtained DNA solution, transformation of the *E. coli* strain JM109 was carried out by the calcium chloride technique. A transformant carrying the desired plasmid (named pGAPhPDILyl) was selected for use in the following vector construction.

##### (B) Construction of a vector for use in the integration of human PDI expression unit into the locus *ura3* on yeast chromosome:

Using the plasmid pGAPhPDILyl prepared above, a vector for use in the integration of human PDI expression unit into yeast chromosome was constructed in the following manner:

Plasmid vector pRS306 (Sikorski and Hierter, 1989) was digested with *Bam*HI and *Xho*I, and the resulting digest was subjected to 0.7% agarose gel electrophoresis to separate a DNA fragment of 4.3 kb which was subsequently purified using Gene Clean. Separately from this, the ADH transcription terminator cassette vector pUC-ATE DNA (Japanese Patent Application Laying-Open (KOKAI) No. 2-117384) was digested with *Bam*HI and *Sal*I, and the resulting digest was subjected to 1% agarose gel electrophoresis to separate a DNA fragment of about 0.4 kb which was subsequently purified using Gene Clean. The thus recovered 0.4 kb DNA fragment was ligated with the above 4.3 kb fragment using T4 DNA ligase to give a cyclized product. The *E. coli* strain XL1-Blue was transformed with the cyclized product to give a transformant carrying the plasmid pRS306-ATE.

Next, the HSA expression vector pRG-UASI-N7-TLY1-305 was digested with *Not*I and *Bam*HI, and the resulting digest was subjected to 0.7% agarose gel electrophoresis to separate a DNA fragment of about 4 kb which was subsequently purified using Gene Clean. Using T4 DNA ligase, the thus recovered DNA fragment was ligated with a *Not*I-*Bam*HI fragment of about 5 kb prepared from the above plasmid pRS306-ATE. Using the cyclized product, transformation of the *E. coli* strain XL1-Blue was carried out to prepare a transformant carrying the HSA expression vector pRG-UASI-N7-TLY1-306.

One  $\mu$ g of the HSA integration vector pRG-UASI-N7-TKLY1-306 DNA prepared by the alkaline technique was digested at 37°C for 2 hours in 30  $\mu$ l of the digestion solution which consists of 10 mM Tris-HCl (pH 0.8), 60 mM NaCl, 7 mM MgCl<sub>2</sub>, 5 units of *Bam*HI (Nippon Gene) and 5 unit of *Hind*III (Nippon Gene). The resulting digest was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 8.5 kb. Separately from this, 2  $\mu$ g of the plasmid, pIVLEUGAPhP-DILyI DNA prepared by the alkaline lysis technique was digested at 37°C for 2 hours in 30  $\mu$ l of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 60 mM NaCl, 7 mM MgCl<sub>2</sub>, 5 units of *Bam*HI (Nippon Gene) and 5 units of *Hin*III (Nippon Gene). The resulting digest was subjected to 0.8% agarose gel electrophoresis and then treated by the glass powder technique to separate and purify a DNA fragment of about 2.5 kb. The two DNA fragments thus prepared (about 50 ng for each) were mixed with the ligation solution (Liquid A 30  $\mu$ l + Liquid B 6  $\mu$ l, Takara Shuzo), and then incubated at 16°C for 1.5 hours so as to ligate the DNA fragments. Using the recombinant plasmid named pIVURAGAPhPDILyI) obtained, transformation of the *E. coli* strain MV1190 was carried out.

#### (C) Introduction of a human PDI expression unit into the locus *ura3* of the HSA-highly secreting yeast strain SN35A:

Using the integration plasmid pIVURAGAPhPDILyI prepared above, transformation of the HSA-highly producing yeast strain SN35A was carried out in the following manner.

30  $\mu$ g of the plasmid pIVURAGAPhPDILyI DNA prepared by the alkaline lysis technique was digested overnight at 37°C in 400  $\mu$ l of the digestion solution which consists of 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 7 mM MgCl<sub>2</sub> and 100 units of *Eco*RV (Takara Shuzo Co., Ltd.). The reaction mixture was subjected to phenol/chloroform extraction and to ethanol precipitation so as to recover the digested DNA fragment which was then dissolved in 10  $\mu$ l of TE buffer.

A single colony of the HSA-highly secreting Strain SN35A was inoculated into 5 ml of YPD medium and cultured overnight at 30°C with shaking. 100  $\mu$ l of the pre-culture was inoculated into 10 ml of the YPD medium, and cultured at 30°C with shaking until turbidity at OD<sub>600</sub> reached about 0.5. The main culture obtained was subjected to centrifugation to recover the yeast cells which were subsequently washed with 1 ml of 0.1 M lithium acetate solution. The cells were then collected by centrifugation, resuspended in 50  $\mu$ l of the 0.1 M lithium acetate solution and then incubated at 30°C for 1 hour. The cell suspension (70  $\mu$ l) was mixed with 10  $\mu$ l of the just described DNA solution, and the mixture was incubated at 30°C for 30 minutes. To the mixture was then added 500  $\mu$ l of the aforementioned PEG solution. After incubation at 30°C for 45 minutes, the mixtures was warmed at 42°C for 5 minutes, mixed with 500  $\mu$ l of sterile water and then subjected to centrifugation to recover the cells. The collected cells were plated on to the SD (-Leu, -His, -Ade, -Ura) agar medium which consists of 20  $\mu$ g/ml of arginine hydrochloride, 20  $\mu$ g/ml of methionine, 20  $\mu$ g/ml of tryptophan, 30  $\mu$ g/ml of isoleucine, 30  $\mu$ g/ml of lysine hydrochloride, 30  $\mu$ g/ml of tyrosine, 50  $\mu$ g/ml of phenylalanine, 100  $\mu$ g/ml of aspartic acid, 100  $\mu$ g/ml of glutamic acid, 150  $\mu$ g/ml of valine, 200  $\mu$ g/ml of threonine, 375  $\mu$ g/ml of serine, 0.67% Bacto-yeast nitrogen base, 2% glucose and 1.5% agar. After the culture of the yeast cells at 30°C for 3 days, a colony grown on the plate was picked up as a transformant which was named SN35A-1PU.

#### Introduction of ERD2 expression unit into the locus TRP1 on the chromosome of the HSA/human PDI co-expression yeast strain, SN35A-1PU

Using the aforementioned integration plasmid pIVTRPADHYERD2, transformation of the HSA/human PDI

co-expression yeast strain SN35A-1PU was carried out in the following manner:

A single colony of the strain SN35A-1PU was inoculated into YPD medium and cultured overnight at 30°C with shaking. 100 µl of the obtained pre-culture was inoculated into 10 ml of the YPD medium, and cultured at 30°C with shaking until turbidity at OD<sub>600</sub> reached about 0.5. The main culture was subjected to centrifugation to recover the yeast cells which were subsequently washed with 1 ml of 0.1 M lithium acetate solution (0.1 M lithium acetate, 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA). The cells were then collected by centrifugation, suspended in 50 µl of the 0.1 M lithium acetate solution and then incubated at 30°C for 1 hour. To the cell suspension (70 µl) was added 30 µg of the ERD2 expression unit integration vector DNA (pIVTRPADHYERD2), and the mixture was incubated at 30°C for 30 minutes. To the mixture was further added 500 µl of a PEG solution (40% polyethylene glycol #4000, 0.1 M lithium acetate, 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA), followed by mixing. After incubation at 30°C for 45 minutes, the cell suspension was warmed at 42°C for 5 minutes, mixed with 500 µl of sterile water and then centrifuged to recover the yeast cells. The cells collected were plated and cultured on the SD (-Leu, -His, -Ade, -Ura, -Trp) plate which consists of 20 µg/ml of arginine hydrochloride, 20 µg/ml of methionine, 30 µg/ml of tyrosine, 30 µg/ml of isoleucine, 30 µg/ml of lysine hydrochloride, 50 µg/ml of phenylalanine, 100 µg/ml of aspartic acid, 100 µg/ml of glutamic acid, 150 µg/ml of valine, 200 µg/ml of threonine, 375 µg/ml of serine, 0.67% Bacto-nitrogen Base, 2% glucose and 1.5% agar. These amino acids used supplied from Wako Pure Chemical Industries. After culturing the cells at 30°C, a colony grown on the plate was picked up as a transformant which was named SN35A-1PUAET.

#### Effect of the co-expression of ERD2 on the intracellular retention of human PDI, and enhancement accompanied thereby of the expression and secretion of HSA

Using the transformant prepared above, effect of the co-expression of ERD2 on the intracellular retention of human PDI and enhancement accompanied thereby of the expression and secretion of HSA were examined in the following manner:

6 single colonies of the transformant SN35A-1PUAET were removed from the SD plate. Each of the colonies was inoculated into 5 ml of the SD (-His, -Leu, -Ade, -Ura, -Trp) medium and precultured at 30°C for 24 hours with shaking. 100 µl of the pre-culture obtained was inoculated into 5 ml of the YPD medium and cultured at 30°C for 24 hours. 200 µl of each culture was centrifuged to recover 100 µl of supernatant which was then mixed the equal volume of ethanol and left for 3 hours on ice. After centrifugation of the precipitates, the pellets collected were separately dried under a reduced pressure, and dissolved in 8 µl of a sample for SDS PAGE (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.72 M β-mercaptoethanol and 0.005% Bromophenol Blue). After boiling for 5 minutes, the samples were subjected to SDS-PAGE on SDS-PAGE Plate 4/20 (Daiichi Kagaku Yakuhin) with 4-20% gradient. At the same time, 0.5 µg of HSA (Sigma) was run as a standard for quantifying a HSA level. The resulting gel was stained with a staining solution (0.15% Coomassie Brilliant Blue, 10% acetic acid and 40% methanol) and then soaked in a decoloring solution (10% acetic acid and 40% methanol) so as to visualize the expressed product in the culture medium. The same procedure was repeated for a main culture sample after culturing for 48 hours, except that 50 µl of the culture was used for the ethanol precipitation. The same procedure was also repeated using the SN35A-1PU as control. Using the gels after SDS-PAGE, the amount of secreted HSA from each strain was determined using a densitometer (IMAGE ANALYSIS SYSTEM, TEFCO). As shown in Figs. 11 and 13, the amount of PDI secreted into the medium decreased sharply due to the co-expression of ERD2 while the secretion of HSA was increased by 26% in average after culturing for 24 hours and by 17% in average after culturing for 48 hours.



5 SEQUENCE LISTING

SEQ ID NO: 1

10 SEQUENCE LENGTH: 2454

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

15 TOPOLOGY: linear

MOLECULE TYPE: cDNA to mRNA

IMMEDIATE SOURCE: human liver and placenta  $\lambda$ gt11 cDNA libraries

20 (Clontech)

**SEQUENCE DESCRIPTION:**

GAATTCGGGG GGCACGAGA GAAGCGCCCC GCCTGATCCG TGTCCGAC ATG CTG CGC 57

Met Leu Arg

-15

CGC GCT CTG CTG TGC CTG GCC GTG GCC GCC CTG GTG CGC GCC GAC GCC 105

30 Arg Ala Leu Leu Cys Leu Ala Val Ala Ala Leu Val Arg Ala Asp Ala

-10

-3-

1

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35    Pro Glu Glu Glu Asp His Val Leu Val Leu Arg Lys Ser Asn Phe. Ala

5

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GAG GCG CTG GCG GCC CAC AAG TAC CTG CTG GTG GAG TTC TAT GCC CCT 201

40    Glu Ala Leu Ala Ala His Lys Tyr Leu Leu Val Glu Phe Tyr Ala Pro

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TGG TGT GGC CAC TGC AAG GCT CTG GCC CCT GAG TAT GCC AAA GCC GCT 249

45 Trp Cys Gly His Cys Lys Ala Leu Ala Pro Glu Tyr Ala Lys Ala Ala

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GGG AAG CTG AAG GCA GAA GGT TCC GAG ATC AGG TTG GCC AAG GTG GAC 297

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5 Gly Lys Leu Lys Ala Glu Gly Ser Glu Ile Arg Leu Ala Lys Val Asp  
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 Ala Thr Glu Glu Ser Asp Leu Ala Gln Gln Tyr Gly Val Arg Gly Tyr  
 70 75 80  
 15 CCC ACC ATC AAG TTC TTC AGG AAT GGA GAC ACG GCT TCC CCC AAG GAA 393  
 Pro Thr Ile Lys Phe Phe Arg Asn Gly Asp Thr Ala Ser Pro Lys Glu  
 85 90 95  
 20 TAT ACA GCT GGC AGA GAG GCT GAT GAC ATC GTG AAC TGG CTG AAG AAG 441  
 Tyr Thr Ala Gly Arg Glu Ala Asp Asp Ile Val Asn Trp Leu Lys Lys  
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 Arg Thr Gly Pro Ala Ala Thr Thr Leu Pro Asp Gly Ala Ala Ala Glu  
 115 120 125 130  
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 Ser Leu Val Glu Ser Ser Glu Val Ala Val Ile Gly Phe Phe Lys Asp  
 135 140 145  
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 Val Glu Ser Asp Ser Ala Lys Gln Phe Leu Gln Ala Ala Glu Ala Ile  
 150 155 160  
 40 GAT GAC ATA CCA TTT GGG ATC ACT TCC AAC AGT GAC GTG TTC TCC AAA 633  
 Asp Asp Ile Pro Phe Gly Ile Thr Ser Asn Ser Asp Val Phe Ser Lys  
 165 170 175  
 45 TAC CAG CTC GAC AAA GAT GGG GTT GTC CTC TTT AAG AAG TTT GAT GAA 681  
 Tyr Gln Leu Asp Lys Asp Gly Val Val Leu Phe Lys Lys Phe Asp Glu  
 180 185 190  
 GGC CGG AAC AAC TTT GAA GGG GAG GTC ACC AAG GAG AAC CTG CTG GAC 729

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5 Gly Arg Asn Asn Phe Glu Gly Glu Val Thr Lys Glu Asn Leu Leu Asp  
 195 200 205 210  
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 10 Phe Ile Lys His Asn Gln Leu Pro Leu Val Ile Glu Phe Thr Glu Gln  
 215 220 225  
 ACA GCC CCG AAG ATT TTT GGA GGT GAA ATC AAG ACT CAC ATC CTG CTG 825  
 15 Thr Ala Pro Lys Ile Phe Gly Gly Glu Ile Lys Thr His Ile Leu Leu  
 230 235 240  
 TTC TTG CCC AAG AGT GTG TCT GAC TAT GAC GGC AAA CTG AGC AAC TTC 873  
 20 Phe Leu Pro Lys Ser Val Ser Asp Tyr Asp Gly Lys Leu Ser Asn Phe  
 245 250 255  
 AAA ACA GCA GCC GAG AGC TTC AAG GGC AAG ATC CTG TTC ATC TTC ATC 921  
 25 Lys Thr Ala Ala Glu Ser Phe Lys Gly Lys Ile Leu Phe Ile Phe Ile  
 260 265 270  
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 30 Asp Ser Asp His Thr Asp Asn Gln Arg Ile Leu Glu Phe Phe Gly Leu  
 275 280 285 290  
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 35 Lys Lys Glu Glu Cys Pro Ala Val Arg Leu Ile Thr Leu Glu Glu Glu  
 295 300 305  
 ATG ACC AAG TAC AAG CCC GAA TCG GAG GAG CTG ACG GCA GAG AGG ATC 1065  
 40 Met Thr Lys Tyr Lys Pro Glu Ser Glu Glu Leu Thr Ala Glu Arg Ile  
 310 315 320  
 ACA GAG TTC TGC CAC CGC TTC CTG GAG GGC AAA ATC AAG CCC CAC CTG 1113  
 45 Thr Glu Phe Cys His Arg Phe Leu Glu Gly Lys Ile Lys Pro His Leu  
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5 Met Ser Gln Glu Leu Pro Glu Asp Trp Asp Lys Gln Pro Val Lys Val  
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 10 Leu Val Gly Lys Asn Phe Glu Asp Val Ala Phe Asp Glu Lys Lys Asn  
 355 360 365 370  
 GTC TTT GTG GAG TTC TAT GCC CCA TGG TGT GGT CAC TGC AAA CAG TTG 1257  
 15 Val Phe Val Glu Phe Tyr Ala Pro Trp Cys Gly His Cys Lys Gln Leu  
 375 380 385  
 GCT CCC ATT TGG GAT AAA CTG GGA GAG ACG TAC AAG GAC CAT GAG AAC 1305  
 20 Ala Pro Ile Trp Asp Lys Leu Gly Glu Thr Tyr Lys Asp His Glu Asn  
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 25 Ile Val Ile Ala Lys Met Asp Ser Thr Ala Asn Glu Val Glu Ala Val  
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 AAA GTG CAC AGC TTC CCC ACA CTC AAG TTC TTT CCT GCC AGT GCC GAC 1401  
 30 Lys Val His Ser Phe Pro Thr Leu Lys Phe Phe Pro Ala Ser Ala Asp  
 420 425 430  
 AGG ACG GTC ATT GAT TAC AAC GGG GAA CGC ACG CTG GAT GGT TTT AAG 1449  
 Arg Thr Val Ile Asp Tyr Asn Gly Glu Arg Thr Leu Asp Gly Phe Lys  
 35 435 440 445 450  
 AAA TTC CTG GAG AGC GGT GGC CAG GAT GGG GCA GGG GAT GAT GAC GAT 1497  
 Lys Phe Leu Glu Ser Gly Gly Gln Asp Gly Ala Gly Asp Asp Asp Asp  
 40 455 460 465  
 CTC GAG GAC CTG GAA GAA GCA GAG GAG CCA GAC ATG GAG GAA GAC GAT 1545  
 Leu Glu Asp Leu Glu Glu Ala Glu Glu Pro Asp Met Glu Glu Asp Asp  
 45 470 475 480  
 GAT CAG AAA GCT GTG AAA GAT GAA CTG TAA TACGCAAAGC CAGACCCGGG 1595

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Asp Gln Lys Ala Val Lys Asp Glu Leu \*

	485	490	
	CGCTGCCGAG	ACCCCTCGGG	GGCTGCACAC CCAGCAGCAG CGCACGCCTC CGAAGCCTGC 1655
	GGCCTCGCTT	GAAGGAGGGC	GTCGCCGGAA ACCCAGGGAA CCTCTCTGAA GTGACACCTC 1715
10	ACCCCTACAC	ACCGTCCGTT	CACCCCCGTC TCTTCCTTCT GCTTTTCGGT TTTTGGAAAG 1775
	GGATCCATCT	CCAGGCAGCC	CACCCTGGTG GGGCTTGTTT CCTGAAACCA TGATGTACTT 1835
	TTTCATACAT	GAGTCTGTCC	AGAGTGCTTG CTACCGTGTI CGGAGTCTCG CTGCCTCCCT 1895
15	CCCGCGGGAG	GTTTCTCCTC	TTTTTGAAAA TTCCGTCTGT GGGATTTTTC GACATTTTTC 1955
	GACATCAGGG	TATTGTTC	ACCTTGGCCA GGCTCCTCG GAGAAGCTTG TCCCCCGTGT 2015
	GGGAGGGACG	GAGCCGGA	CTGGACATGGTC ACTCAGTACC GCCTGCAGTG TCGCCATGAC 2075
20	TGATCATGGC	TCTTGCA	TTGGGTAAT GGAGACTTCC GGATCCTGTC AGGGTGTCCC 2135
	CCATGCCTGG	AAGAGGAGCT	GGTGGCTGCC AGCCCTGGGG CCCGGCACAG GCCTGGGCCT 2195
	TCCCCTTCCC	TCAAGCCAGG	GCTCCTCCTC CTGTCGTGGG CTCATTGTGA CCACTGGCCT 2255
25	CTCTACAGCA	CGGCCTGTGG	CCTGTTCAAG GCAGAACCAC GACCCTTGAC TCCCGGGTGG 2315
	GGAGGTGGCC	AAGGATGCTG	GAGCTGAATC AGACGCTGAC AGTTCTTCAG GCATTTCTAT 2375
	TTCACAATCG	AATTGAACAC	ATTGGCCAAA TAAAGTTGAA ATTTTACCCA CCCAAAAAA 2435
30	AAAAAAAAAA	CCCGAATTC	2454

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SEQ ID NO: 2

SEQUENCE LENGTH: 1545

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SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

**TOPOLOGY:** linear

45

MOLECULE TYPE: other nucleic acid (semi-synthetic DNA)

**SEQUENCE DESCRIPTION:**

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ATG AAG TGG GTT ACC TTC ATC TCT TTG TTG 30  
Met Lys Trp Val Thr Phe Ile Ser Leu Leu

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5		-20	-15	
	TTC TTG TTC TCT TCT GCT TAC TCT AGA GGT GTT TTC AGA AGG GGC GCC	78		
	Phe Leu Phe Ser Ser Ala Tyr Ser Arg Gly Val Phe Arg Arg Gly Ala			
10		-10	-5	1
	CCC GAG GAG GAG GAC CAC GTC CTG GTG CTG CGG AAA AGC AAC TTC GCG	126		
	Pro Glu Glu Glu Asp His Val Leu Val Leu Arg Lys Ser Asn Phe Ala			
15		5	10	15
	GAG GCG CTG GCG GCC CAC AAG TAC CTG CTG GTG GAG TTC TAT GCC CCT	174		
	Glu Ala Leu Ala Ala His Lys Tyr Leu Leu Val Glu Phe Tyr Ala Pro			
20		20	25	30
	TGG TGT GGC CAC TGC AAG GCT CTG GCC CCT GAG TAT GCC AAA GCC GCT	222		
	Trp Cys Gly His Cys Lys Ala Leu Ala Pro Glu Tyr Ala Lys Ala Ala			
25		35	40	45
	GGG AAG CTG AAG GCA GAA GGT TCC GAG ATC AGG TTG GCC AAG GTG GAC	270		
	Gly Lys Leu Lys Ala Glu Gly Ser Glu Ile Arg Leu Ala Lys Val Asp			
30		55	60	65
	GCC ACG GAG GAG TCT GAC CTG GCC CAG CAG TAC GGC GTG CGC GGC TAT	318		
	Ala Thr Glu Glu Ser Asp Leu Ala Gln Gln Tyr Gly Val Arg Gly Tyr			
35		70	75	80
	CCC ACC ATC AAG TTC TTC AGG AAT GGA GAC ACG GCT TCC CCC AAG GAA	366		
	Pro Thr Ile Lys Phe Phe Arg Asn Gly Asp Thr Ala Ser Pro Lys Glu			
40		85	90	95
	TAT ACA GCT GGC AGA GAG GCT GAT GAC ATC GTG AAC TGG CTG AAG AAG	414		
	Tyr Thr Ala Gly Arg Glu Ala Asp Asp Ile Val Asn Trp Leu Lys Lys			
45		100	105	110
	CGC ACG GGC CCG GCT GCC ACC ACC CTG CCT GAC GGC GCA GCT GCA GAG	462		
	Arg Thr Gly Pro Ala Ala Thr Thr Leu Pro Asp Gly Ala Ala Ala Glu			
50				
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5	115	120	125	130	
	TCC TTG GTG GAG TCC AGC GAG GTG GCT GTC ATC GGC TTC TTC AAG GAC	510			
	Ser Leu Val Glu Ser Ser Glu Val Ala Val Ile Gly Phe Phe Lys Asp				
10	135	140	145		
	GTG GAG TCG GAC TCT GCC AAG CAG TTT TTG CAG GCA GCA GAG GCC ATC	558			
	Val Glu Ser Asp Ser Ala Lys Gln Phe Leu Gln Ala Ala Glu Ala Ile				
15	150	155	160		
	GAT GAC ATA CCA TTT GGG ATC ACT TCC AAC AGT GAC GTG TTC TCC AAA	606			
	Asp Asp Ile Pro Phe Gly Ile Thr Ser Asn Ser Asp Val Phe Ser Lys				
20	165	170	175		
	TAC CAG CTC GAC AAA GAT GGG GTT GTC CTC TTT AAG AAG TTT GAT GAA	654			
	Tyr Gln Leu Asp Lys Asp Gly Val Val Leu Phe Lys Lys Phe Asp Glu				
25	180	185	190		
	GGC CGG AAC AAC TTT GAA GGG GAG GTC ACC AAG GAG AAC CTG CTG GAC	702			
	Gly Arg Asn Asn Phe Glu Gly Glu Val Thr Lys Glu Asn Leu Leu Asp				
30	195	200	205	210	
	TTT ATC AAA CAC AAC CAG CTG CCC CTT GTC ATC GAG TTC ACC GAG CAG	750			
	Phe Ile Lys His Asn Gln Leu Pro Leu Val Ile Glu Phe Thr Glu Gln				
35	215	220	225		
	ACA GCC CCG AAG ATT TTT GGA GGT GAA ATC AAG ACT CAC ATC CTG CTG	798			
	Thr Ala Pro Lys Ile Phe Gly Gly Glu Ile Lys Thr His Ile Leu Leu				
40	230	235	240		
	TTC TTG CCC AAG AGT GTG TCT GAC TAT GAC GGC AAA CTG AGC AAC TTC	846			
	Phe Leu Pro Lys Ser Val Ser Asp Try Asp Gly Lys Leu Ser Asn Phe				
45	245	250	255		
	AAA ACA GCA GCC GAG AGC TTC AAG GGC AAG ATC CTG TTC ATC TTC ATC	894			
	Lys Thr Ala Ala Glu Ser Phe Lys Gly Lys Ile Leu Phe Ile Phe Ile				

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5	260	265	270	
	GAC AGC GAC CAC ACC GAC AAC CAG CGC ATC CTC GAG TTC TTT GGC CTG	942		
	Asp Ser Asp His Thr Asp Asn Gln Arg Ile Leu Glu Phe Phe Gly Leu			
10	275	280	285	290
	AAG AAG GAA GAG TGC CCG GCC GTG CGC CTC ATC ACC CTG GAG GAG GAG	990		
	Lys Lys Glu Glu Cys Pro Ala Val Arg Leu Ile Thr Leu Glu Glu Glu			
15		295	300	305
	ATG ACC AAG TAC AAG CCC GAA TCG GAG GAG CTG ACG GCA GAG AGG ATC	1038		
	Met Thr Lys Tyr Lys Pro Glu Ser Glu Glu Leu Thr Ala Glu Arg Ile			
20		310	315	320
	ACA GAG TTC TGC CAC CGC TTC CTG GAG GGC AAA ATC AAG CCC CAC CTG	1086		
	Thr Glu Phe Cys His Arg Phe Leu Glu Gly Lys Ile Lys Pro His Leu			
25		325	330	335
	ATG AGC CAG GAG CTG CCG GAG GAC TGG GAC AAG CAG CCT GTC AAG GTG	1134		
	Met Ser Gln Glu Leu Pro Glu Asp Trp Asp Lys Gln Pro Val Lys Val			
30		340	345	350
	CTT GTT GGG AAG AAC TTT GAA GAC GTG GCT TTT GAT GAG AAA AAA AAC	1182		
	Leu Val Gly Lys Asn Phe Glu Asp Val Ala Phe Asp Glu Lys Lys Asn			
35		355	360	365
	GTC TTT GTG GAG TTC TAT GCC CCA TGG TGT GGT CAC TGC AAA CAG TTG	1230		
	Val Phe Val Glu Phe Tyr Ala Pro Trp Cys Gly His Cys Lys Gln Leu			
40		375	380	385
	GCT CCC ATT TGG GAT AAA CTG GGA GAG ACG TAC AAG GAC CAT GAG AAC	1278		
	Ala Pro Ile Trp Asp Lys Leu Gly Glu Thr Tyr Lys Asp His Glu Asn			
45		390	395	400
	ATC GTC ATC GCC AAG ATG GAC TCG ACT GCC AAC GAG GTG GAG GCC GTC	1326		
	Ile Val Ile Ala Lys Met Asp Ser Thr Ala Asn Glu Val Glu Ala Val			

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      405              410              415
5  AAA GTG CAC AGC TTC CCC ACA CTC AAG TTC TTT CCT GCC AGT GCC GAC 1374
   Lys Val His Ser Phe Pro Thr Leu Lys Phe Phe Pro Ala Ser Ala Asp

      420              425              430
10 AGG ACG GTC ATT GAT TAC AAC GGG GAA CGC ACG CTG GAT GGT TTT AAG 1422
   Arg Thr Val Ile Asp Tyr Asn Gly Glu Arg Thr Leu Asp Gly Phe Lys

      435              440              445              450
15 AAA TTC CTG GAG AGC GGT GGC CAG GAT GGG GCA GGG GAT GAT GAC GAT 1470
   Lys Phe Leu Glu Ser Gly Gly Gln Asp Gly Ala Gly Asp Asp Asp Asp

      455              460              465
20 CTC GAG GAC CTG GAA GAA GCA GAG GAG CCA GAC ATG GAG GAA GAC GAT 1518
   Leu Glu Asp Leu Glu Glu Ala Glu Glu Pro Asp Met Glu Glu Asp Asp

      470              475              480
25 GAT CAG AAA GCT GTG AAA GAT GAA CTG 1545
   Asp Gln Lys Ala Val Lys Asp Glu Leu

      485              490

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SEQ ID NO: 3

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SEQUENCE LENGTH: 491

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

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MOLECULAR TYPE: protein

SEQUENCE DESCRIPTION:

Gly Ala Pro Glu Glu Glu Asp His Val Leu Val Leu Arg Lys Ser Asn

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Phe Ala Glu Ala Leu Ala Ala His Lys Tyr Leu Leu Val Glu Phe Tyr

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Ala Pro Trp Cys Gly His Cys Lys Ala Leu Ala Pro Glu Tyr Ala Lys  
 5                    35                    40                    45  
 Ala Ala Gly Lys Leu Lys Ala Glu Gly Ser Glu Ile Arg Leu Ala Lys  
                   50                    55                    60  
 10 Val Asp Ala Thr Glu Glu Ser Asp Leu Ala Gln Gln Tyr Gly Val Arg  
                   65                    70                    75                    80  
 15 Gly Tyr Pro Thr Ile Lys Phe Phe Arg Asn Gly Asp Thr Ala Ser Pro  
                   85                    90                    95  
 Lys Glu Tyr Thr Ala Gly Arg Glu Ala Asp Asp Ile Val Asn Trp Leu  
 20                    100                    105                    110  
 Lys Lys Arg Thr Gly Pro Ala Ala Thr Thr Leu Pro Asp Gly Ala Ala  
                   115                    120                    125  
 25 Ala Glu Ser Leu Val Glu Ser Ser Glu Val Ala Val Ile Gly Phe Phe  
                   130                    135                    140  
 Lys Asp Val Glu Ser Asp Ser Ala Lys Gln Phe Leu Gln Ala Ala Glu  
 30                    145                    150                    155                    160  
 Ala Ile Asp Asp Ile Pro Phe Gly Ile Thr Ser Asn Ser Asp Val Phe  
                   165                    170                    175  
 35 Ser Lys Tyr Gln Leu Asp Lys Asp Gly Val Val Leu Phe Lys Lys Phe  
                   180                    185                    190  
 Asp Glu Gly Arg Asn Asn Phe Glu Gly Glu Val Thr Lys Glu Asn Leu  
 40                    195                    200                    205  
 Leu Asp Phe Ile Lys His Asn Gln Leu Pro Leu Val Ile Glu Phe Thr  
 45                    210                    215                    220  
 Glu Gln Thr Ala Pro Lys Ile Phe Gly Gly Glu Ile Lys Thr His Ile  
                   225                    230                    235                    240  
 50 Leu Leu Phe Leu Pro Lys Ser Val Ser Asp Tyr Asp Gly Lys Leu Ser

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	245	250	255
5	Asn Phe Lys Thr Ala Ala Glu Ser Phe Lys Gly Lys Ile Leu Phe Ile		
	260	265	270
	Phe Ile Asp Ser Asp His Thr Asp Asn Gln Arg Ile Leu Glu Phe Phe		
10	275	280	285
	Gly Leu Lys Lys Glu Glu Cys Pro Ala Val Arg Leu Ile Thr Leu Glu		
	290	295	300
15	Glu Glu Met Thr Lys Tyr Lys Pro Glu Ser Glu Glu Leu Thr Ala Glu		
	305	310	315
	Arg Ile Thr Glu Phe Cys His Arg Phe Leu Glu Gly Lys Ile Lys Pro		
20	325	330	335
	His Leu Met Ser Gln Glu Leu Pro Glu Asp Trp Asp Lys Gln Pro Val		
	340	345	350
25	Lys Val Leu Val Gly Lys Asn Phe Glu Asp Val Ala Phe Asp Glu Lys		
	355	360	365
30	Lys Asn Val Phe Val Glu Phe Tyr Ala Pro Trp Cys Gly His Cys Lys		
	370	375	380
	Gln Leu Ala Pro Ile Trp Asp Lys Leu Gly Glu Thr Tyr Lys Asp His		
35	385	390	395
	Glu Asn Ile Val Ile Ala Lys Met Asp Ser Thr Ala Asn Glu Val Glu		
	405	410	415
40	Ala Val Lys Val His Ser Phe Pro Thr Leu Lys Phe Phe Pro Ala Ser		
	420	425	430
	Ala Asp Arg Thr Val Ile Asp Tyr Asn Gly Glu Arg Thr Leu Asp Gly		
45	435	440	445
	Phe Lys Lys Phe Leu Glu Ser Gly Gly Gln Asp Gly Ala Gly Asp Asp		
50	450	455	460

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Asp Asp Leu Glu Asp Leu Glu Glu Ala Glu Glu Pro Asp Met Glu Glu

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Asp Asp Asp Gln Lys Ala Val Lys Asp Glu Leu

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# Claims

1. A transformant comprising the following expression units integrated on yeast chromosome in a co-expressible state:
  - an expression unit containing a gene coding for a receptor protein ERD2 from yeast or analog thereof which is capable of binding to a protein localizing in endoplasmic reticulum and having a signal for staying therein; and
  - an expression unit containing a gene coding for said protein localizing in endoplasmic reticulum and having a signal for staying therein.
2. A transformant according to claim 1, wherein said expression unit containing a gene coding for a receptor protein ERD2 from yeast or analog thereof is expression plasmid pIVTRPADHYERD2.
3. A transformant according to claim 1, wherein said gene coding for a protein localizing in endoplasmic reticulum and having a signal for staying therein is a protein disulfide isomerase gene, or a fusion which is composed of a DNA fragment coding for a human serum albumin prepro-sequence and the isomerase gene.
4. A transformant according to claim 3, wherein said fusion gene has a DNA sequence coding for the -24 +491 amino acid sequence shown in SEQ ID NO:2.
5. A transformant according to claim 4, wherein said DNA sequence is the sequence between nucleotide 1 and nucleotide 1545 shown in SEQ ID NO:2.
6. A transformant according to claim 3, wherein said protein disulfide isomerase (PDI) gene is a human PDI gene or its derivative.
7. A transformant comprising the following expression units integrated on yeast chromosome in a co-expressible state:
  - an expression unit containing a gene coding for a receptor protein ERD2 from yeast or analog thereof which is capable of binding to a protein localizing in endoplasmic reticulum and having a signal for staying therein;
  - an expression unit containing a gene coding for said protein localizing in endoplasmic reticulum and having a signal for staying therein; and
  - an expression unit containing a foreign gene coding for a polypeptide which is a subject of function of said protein localizing in endoplasmic reticulum and having a signal for staying therein.
8. A transformant according to claim 7, wherein said expression unit containing a gene coding for a receptor protein ERD2 from yeast or analog thereof is expression plasmid pIVTRPAHYERD2.
9. A transformant according to claim 7, wherein said gene coding for a protein localizing in endoplasmic reticulum and having a signal staying therein is a protein disulfide isomerase gene, or a fusion gene which is composed of a DNA fragment coding for a human serum albumin prepro-sequence and said isomerase gene.
10. A transformant according to claim 9, wherein said fusion gene has a DNA sequence coding for the -24 to +491 amino acid sequence shown in SEQ ID NO:2.

11. A transformant according to claim 10, wherein said DNA sequence is the sequence between nucleotide I and nucleotide 1545 shown in SEQ ID NO:2.
12. A transformant according to claim 9, wherein said protein disulfite isomerase (PDI) gene is a human PDI gene or its derivative.
13. A transformant according to claim 7, wherein said foreign gene is a gene coding for human serum albumin.
14. A transformant according to claim 7, which is a yeast strain SN35A-1PUAET.
15. A fusion gene for use in an expression of human protein disulfide isomerase, which is composed of a DNA fragment coding for a human serum albumin prepro-sequence and a gene coding for said isomerase.
16. A fusion gene according to claim 15, which has a DNA sequence coding for the -24 to +491 amino acid sequence shown in SEQ ID NO:2.
17. A fusion gene according to claim 16, wherein said DNA sequence is the sequence between nucleotide I and nucleotide 1545 shown in SEQ ID NO:2.
18. A transformant comprising, in a co-expressible state, the fusion gene according to any of the claims 15 to 17 and a foreign gene coding for a polypeptide to be produced.
19. A transformant according to claim 18, which is a transformed yeast cell.
20. A transformant according to claim 18, wherein said foreign gene is a gene coding for human serum albumin.
21. A process for producing a polypeptide, which comprises the following steps of:  
culturing the transformant according to any one of the claims 7 to 14 in an appropriate medium, and bringing about co-expression such that the polypeptide is predominantly secreted out of the transformant cell, the polypeptide being a subject of function of a protein which localizes in endoplasmic reticulum and has a signal for staying therein, while both a receptor protein ERD2 from yeast or analog thereof which is capable of binding to said protein localizing in endoplasmic reticulum, and said protein as a ligand for the ERD2 stay in endoplasmic reticulum; and  
recovering said polypeptide secreted.
22. A process according to claim 21, wherein said polypeptide is human serum albumin.
23. A process for producing a polypeptide, which comprises the following steps of:  
co-expressing a human protein disulfide isomerase gene and a foreign gene coding for the polypeptide to be produced, in the transformant according to any one of claims 18 to 20 so as to produce the polypeptide; and  
recovering said polypeptide.
24. A process according to claim 23, wherein said polypeptide is human serum albumin.

*Fig. 1(i)*

Fig. 1

Fig. 1(I)
Fig. 1(II)
Fig. 1(III)

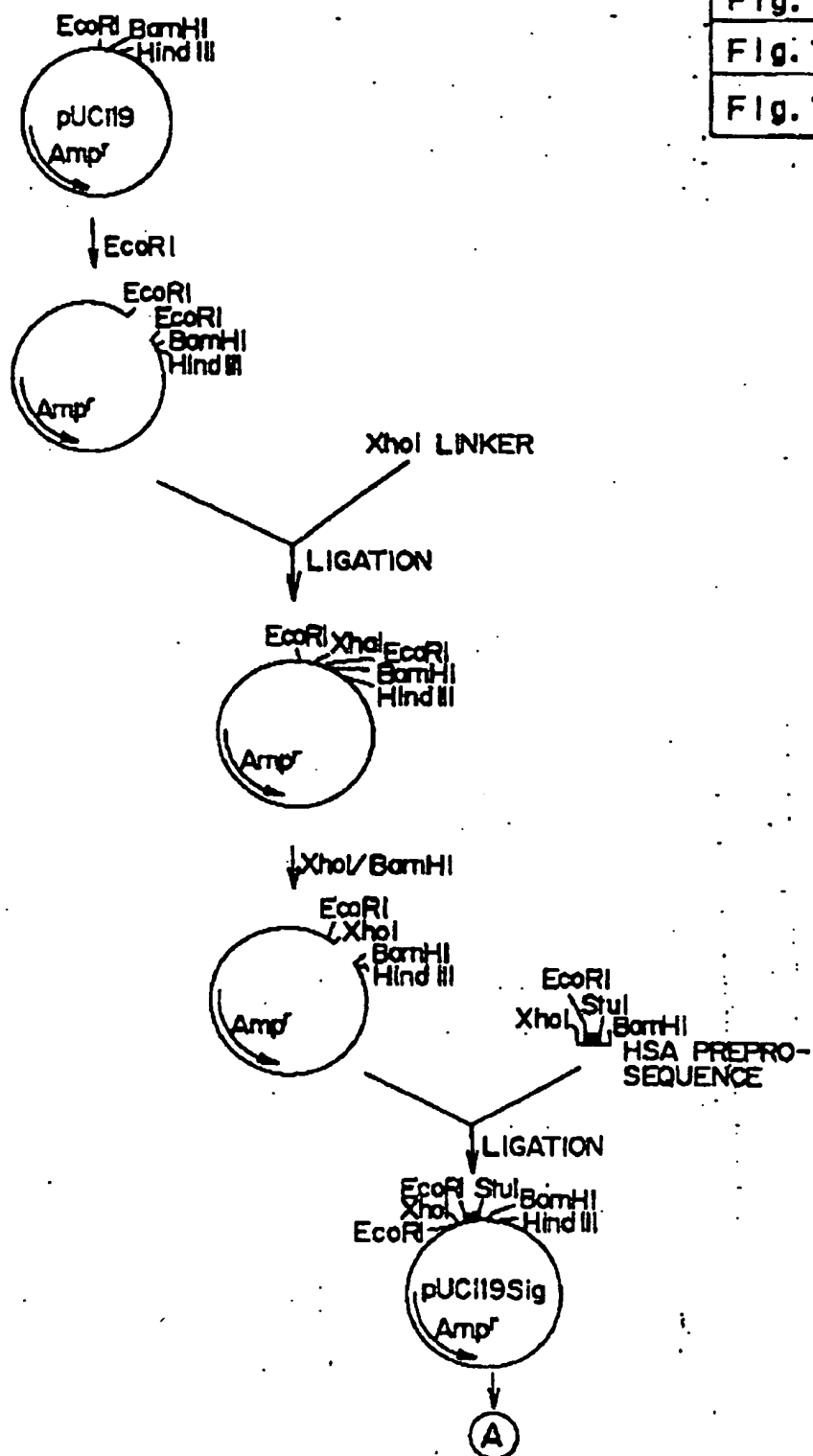




Fig. 1 (iii)

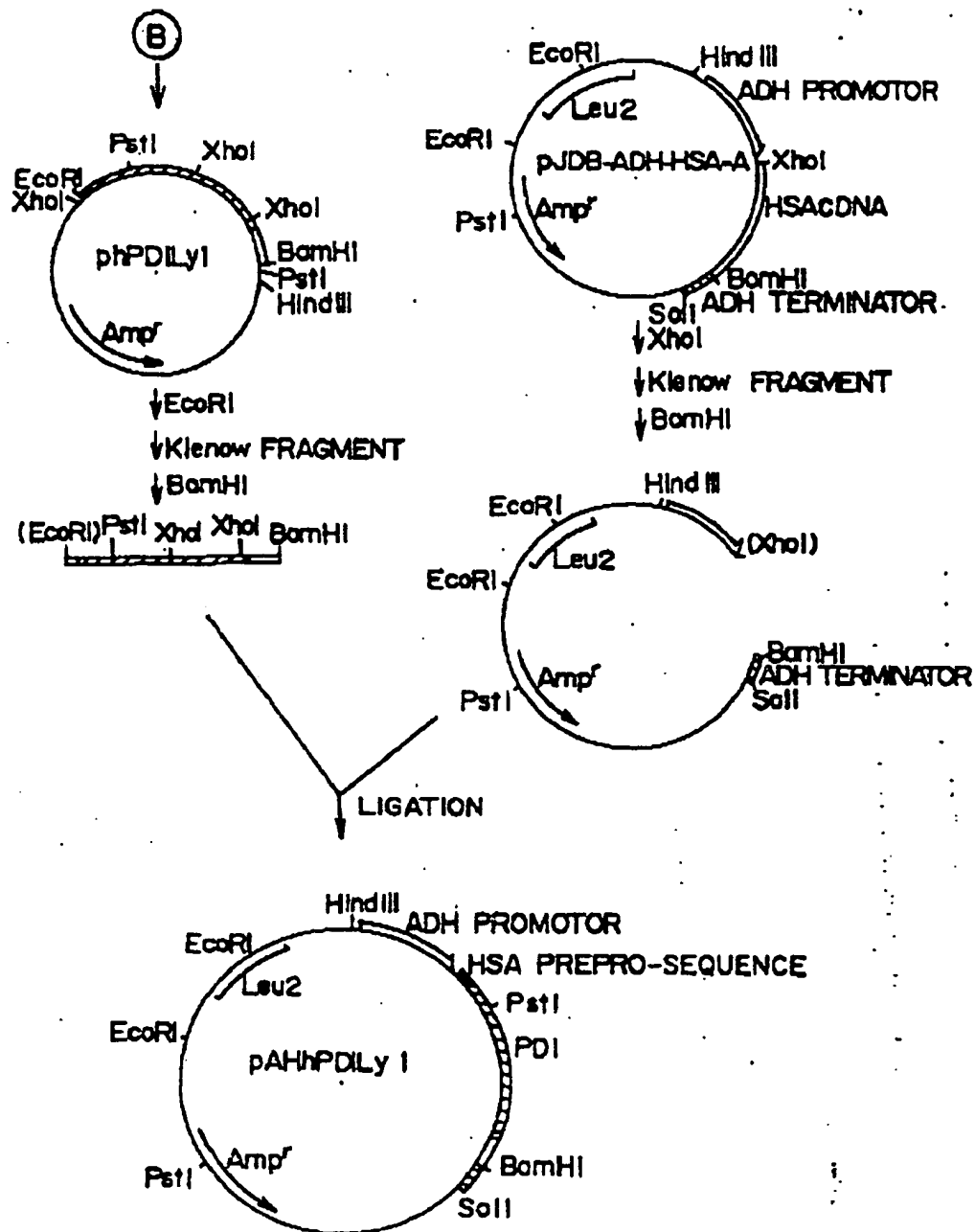




Fig. 2

HSA PREPRO-SEQUENCE PD1

---ATG AAG TGGGTAACTTCACTTTTGTTGTTCTTCTTCTTCTAGAAGGCGCCGCCGAGGAGGACGAC---

.....Met Lys . Trp Val Thr Phe Ile Ser Leu Leu Phe Ser Ala Tyr Ser Arg Gly Val Phe Arg Arg Gly Ala Pro Glu Glu Asp His...

*Fig. 3*

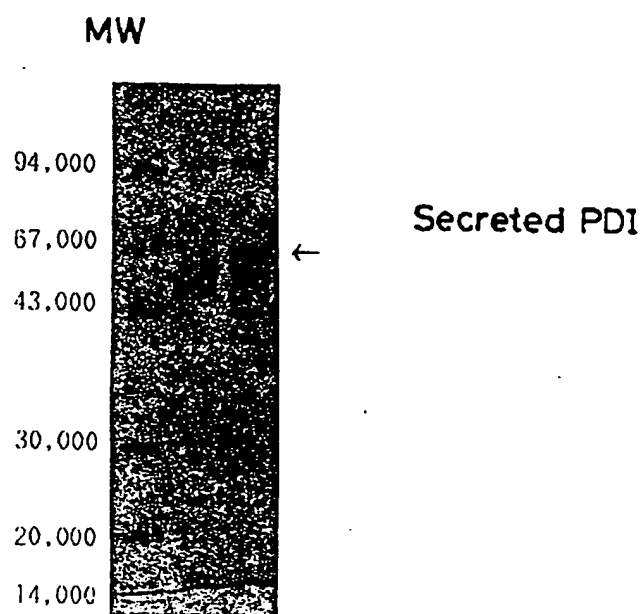
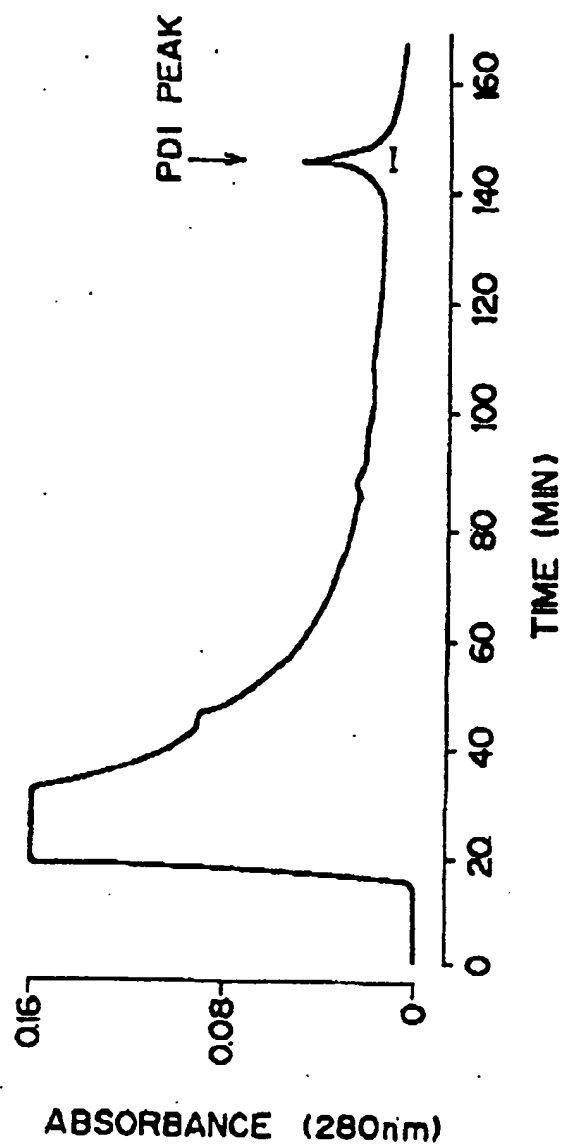
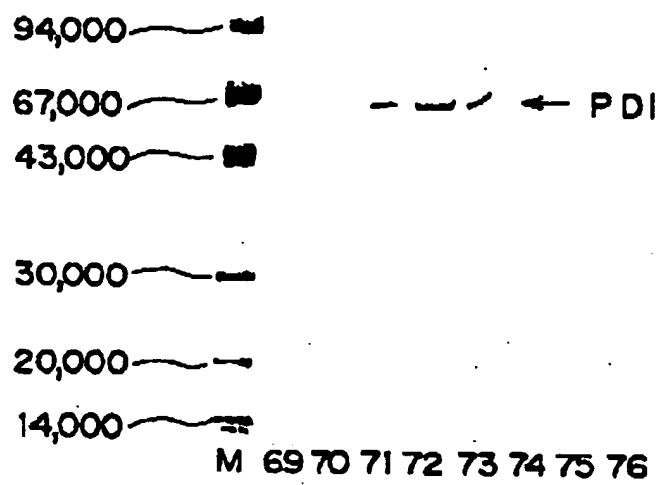


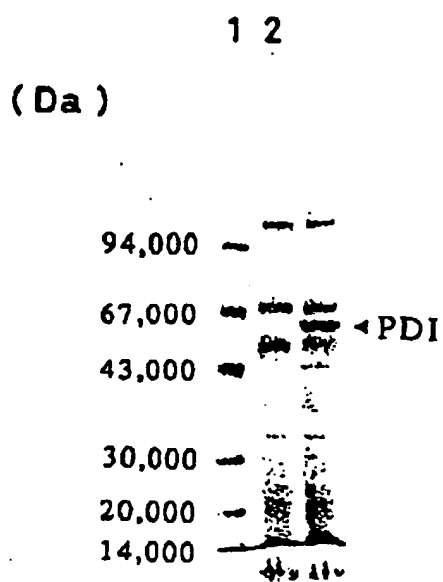
Fig. 4



*Fig. 5*



*Fig. 6*



1: pAH/HIS23

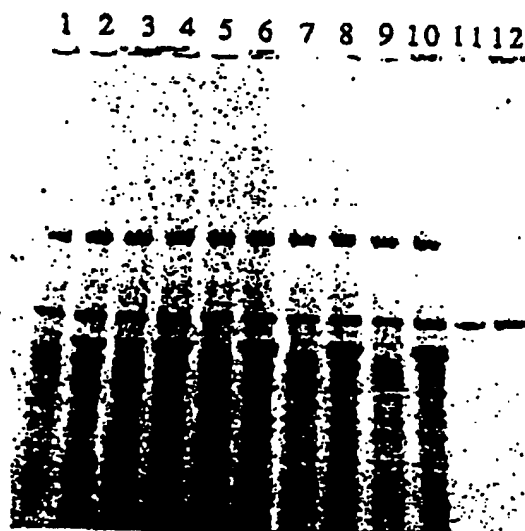
2: pAHhPDILy1/HIS23

*Fig. 7*

1 2

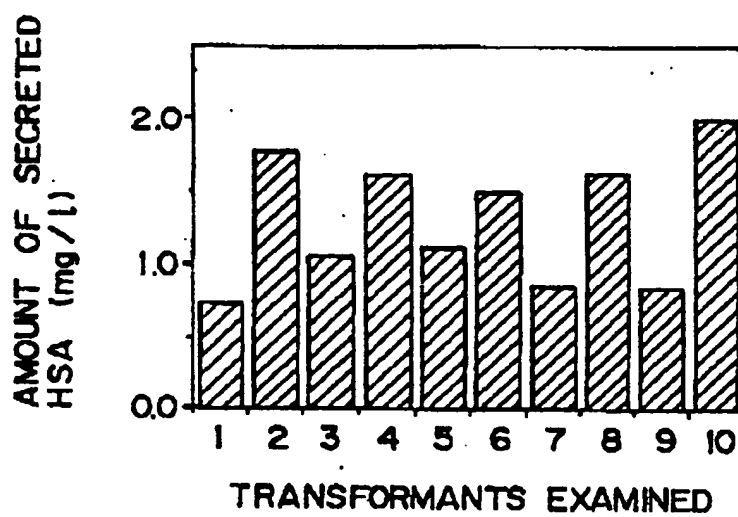
Secreted HSA  
Secreted PDI

HSA ▶



1, 3, 5, 7, 9: pAH/HIS 23  
2, 4, 6, 8, 10: pAHhPDILy1/HIS 23  
11: HSA STANDARD 0.25ug  
12: HSA STANDARD 0.5ug

*Fig. 8*



1, 3, 5, 7, 9: pAH/HIS23

2, 4, 6, 8, 10: pAHhPDILy1/HIS23

*Fig. 9*

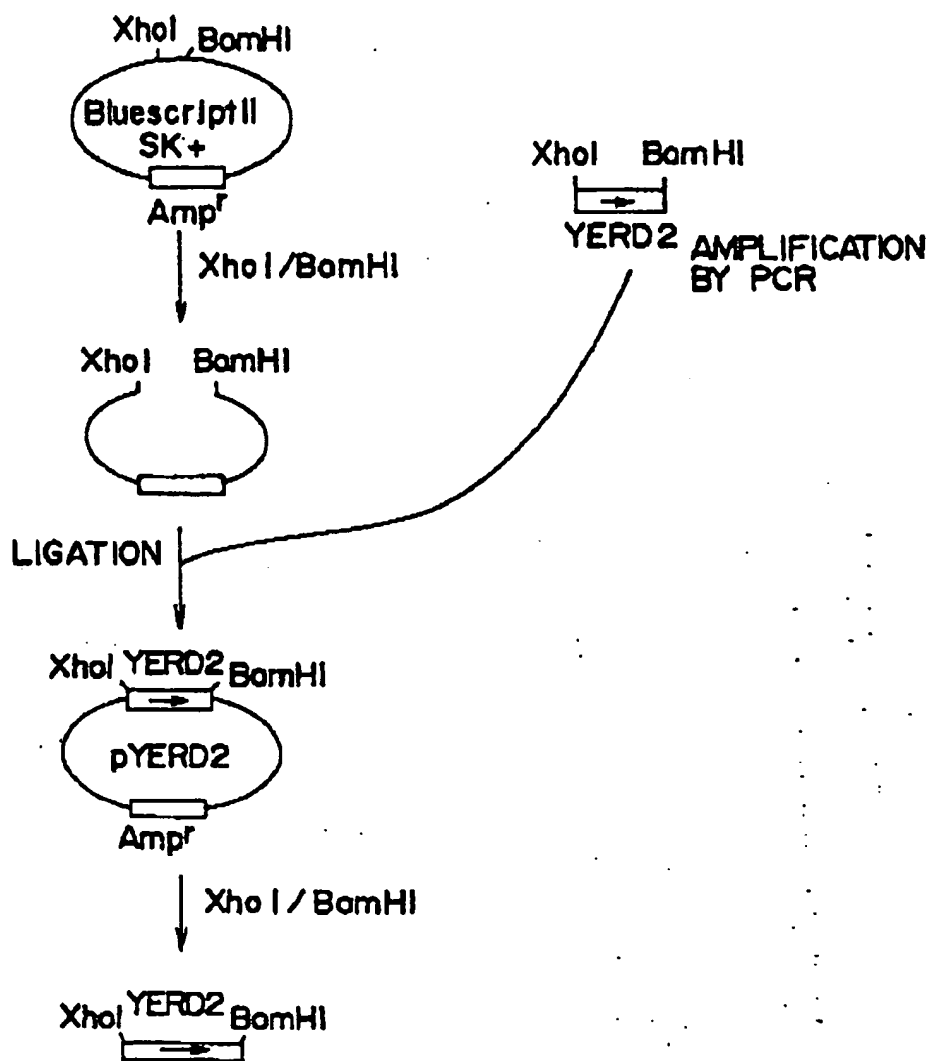
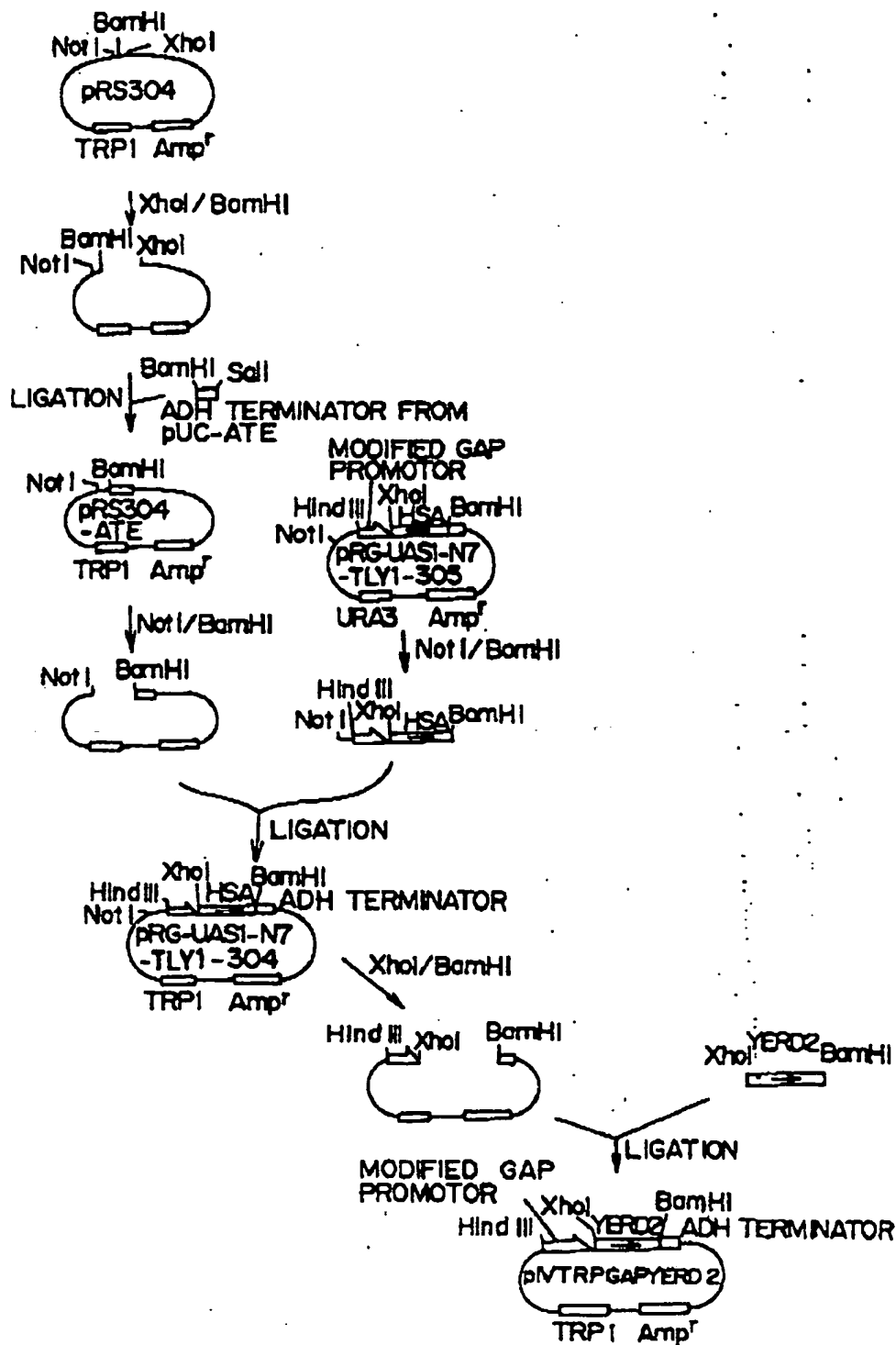
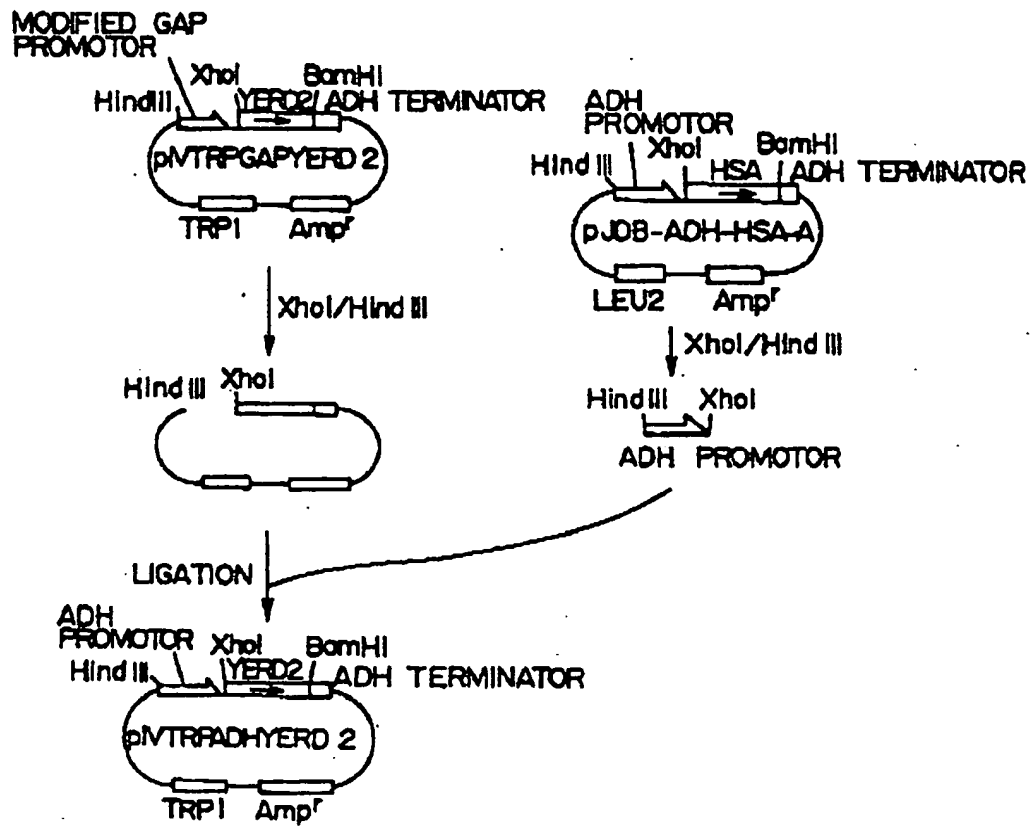




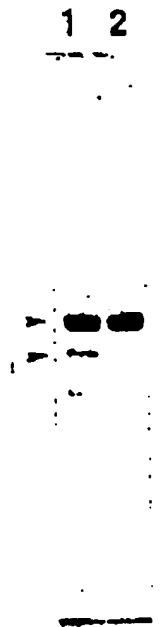
Fig. 10



*Fig. 11*



*Fig. 12*



*Fig. 13*